(54) Title: AN ASSAY REAGENT AND A METHOD OF MAKING AND USING THE ASSAY REAGENT

(57) Abstract

An assay compound or a salt thereof for assaying the activity of an enzyme inside a metabolically active whole cell is disclosed. The assay compound includes a leaving group and an indicator group. The leaving group is selected from the group comprising amino acids, peptides, saccharides, sulfates, phosphates, esters, phosphate esters, nucleotides, polynucleotides, nucleic acids, pyrimidines, purines, nucleosides, lipids and mixtures thereof. The indicator group is selected from compounds which have a first state when joined to the leaving group, and a second state when the leaving group is cleaved from the indicator group by the enzyme. Preferably, the indicator compounds are rhodamine 110, rhodol, and fluorescein and analogs of these compounds. A method of synthesizing the compound as well as methods of using these compounds to measure enzyme activity are also disclosed.

AN ASSAY REAGENT AND A METHOD
OF MAKING AND USING THE ASSAY REAGENT

BACKGROUND OF THE INVENTION
Field of the Invention

5   This invention relates generally to cytoenzymology,
and more particularly reagents for use in cytoenzymology
as well as production and use of these reagents.

Description of the Background Art

Cytoenzymology is the study of enzymes as they
10   function on and within cells. Previously, the study of
enzymatic activity within cells has been pursued
primarily by two indirect methods. According to a first
method, the cell membrane is broken to create a cytosol
of cellular components including the enzyme which is the
15   object of study. Various tests are then performed to
determine the activity of the enzyme, which tests can be
performed on the cytosol or on the purified enzyme.
According to a second method, the enzyme activity is
determined from the study of extra-cellular events, such
20   as the presence or lack of the products of enzyme
activity.

According to the first method, various tests are
performed to determine enzyme activity in the cytosol.
One such test is to provide a substrate that is
25   recognized by the enzyme, with a fluorescent compound
which will undergo a detectable change when the
substrate, or "leaving group", is cleaved from the
compound by the enzyme. Mangel et al., U.S. Patent Nos.

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4,557,862 and 4,640,893, disclose rhodamine 110-based derivatives as fluorogenic substrates for proteinases. These compounds have the general formula:

$$(Cbz\text{-}peptide\text{-}NH)_2\text{-}rhodamine\ 110$$
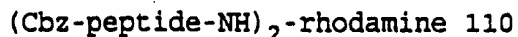
5   where the peptide includes known amino acids or amino acid derivatives, and "Cbz" refers to the blocking group benzyloxycarbonyl. When the amino groups of rhodamine 110 are blocked the compound is "quenched", and is relatively colorless and non-fluorescent. Cleavage of

10   one of the peptides from the non-fluorescent bisamide substrate results in a 3500-fold increase in fluorescence intensity.

The rhodamine 110 substrates of Mangel et al. have been utilized to conduct cytoenzymological studies. G.

15   Rothe et al., Biol. Chem. Hoppe-Seyler, 373, 544-547 (1992) describe the analysis of proteinase activities using the substituted peptide-rhodamine 110 derivatives of Mangel et al. Moreover, G. Valet et al, Ann NY Acad Sci, 667, 233-251 (1993), disclose the study of white

20   cell and thrombocyte disorders with the rhodamine 110 derivatives of Mangel et al. The methods of Rothe and Valet have been used to conduct cytoenzymological studies on the activity of enzymes with cells, but the compounds utilized by Rothe and Valet are not suitable

25   for the study of the activity of intracellular enzymes in vital cells. The Mangel et al. compounds cannot be efficiently solubilized and transmitted through the cell membrane in a manner which will produce a reliable assay. In addition, the Cbz group in the Mangel et al.

30   compound is not recognized by the enzyme's active sites. Further, Mangel, et al., disclose the removal of the carbobenzyloxy group by treating the blocked peptide-indicator compound with 30% hydrobromide acid in acetic acid. However, the bromide salt is lethal to the cell

35   and does not permit an assay for a metabolically active cell.

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I. Mononen, et al., Clin. Chem., 40(3), 385-388
(1994), describe the enzymatic diagnosis of
aspartylglycosaminuria by the fluorometric assay of
glycosylasparaginase in serum, plasma, and lymphocytes.
The study was conducted on cytosols, and not whole
cells, and utilized an asparagine-substituted 7-amino-4-
methylcoumarin.

Dead or metabolically inactive cells can have as
little as approximately one-quarter the enzymatic
activity of living cells, Watson, J., "Enzyme Kinetic
Studies in Cell Population Using Fluorogenic Substrates
and Flow Cytometric Techniques", Cytometry, 1(2), p.
143 (1980). Further, because enzymes are frequently
bound in highly organized enzyme pathways, the
disruption and death of the cell can greatly affect
enzyme activity. Current assays therefore have limited
utility for determining enzyme activity in a living or
metabolically active whole cell.

U.S. Patent 5,070,012 to Nolan et al., describes a
method of monitoring cells and trans-acting
transcription elements. This method, however, is not
designed for the monitoring of enzymes which are
endogenous to the cell being tested. Rather, in this
method a hypotonic solution is used to increase the
permeability of the cell membrane thereby allowing an
exogenous enzyme and other reagents (including a
fluorogenic substrate) to be introduced into the cell.
However, these severe hypotonic conditions significantly
alter the normal state of the cell. The fluorogenic
substrate described in this patent (fluoroscein
digalactopyranoside) contains significant amounts of
fluorescent impurities and must be bleached with a laser
prior to use.

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SUMMARY OF THE INVENTION

The present invention relates to an assay reagent
for determining the activity of an enzyme in a
metabolically active whole cell, said assay reagent
5   comprising at least one water soluble assay compound
having the ability to pass through a cell membrane or a
water soluble physiologically acceptable salt thereof
having the ability to pass through a cell membrane, said
assay compound having a leaving group selected for
10   cleavage by an enzyme to be analyzed and a fluorogenic
indicator group being selected for its ability to have
a non-fluorescent first state when joined to the leaving
group, and a fluorescent second state excitable at a
wavelength above 450 nm when the leaving group is
15   cleaved from the indicator group by the enzyme, said
assay reagent having a fluorescence less than the auto-
fluorescence of a metabolically active cell and being
stable for a minimum of 30 days when stored at 4°C,
wherein said stability is defined as the compound having
20   an increase in background fluorescence of $\leq$ 10%.

The present invention also relates to an assay
reagent for determining the activity of an enzyme in a
metabolically active whole cell, said assay reagent
comprising at least one water soluble salt of an assay
25   compound having the ability to pass through a cell
membrane, said assay compound having a leaving group
selected for cleavage by an enzyme to be analyzed and a
fluorogenic indicator group being selected for its
ability to have a first non-fluorescent state when
30   joined to the leaving group, and a second fluorescent
state excitable at a wavelength above 450 nm when the
leaving group is cleaved from the indicator group by the
enzyme, and said assay reagent having a fluorescence
less than the auto-fluorescence of a metabolically
35   active cell.

5

The present invention also relates to an assay
reagent composition for determining the activity of an
enzyme in a metabolically active whole cell, said assay
reagent comprising at least one water soluble assay
5   compound having the ability to pass through a cell
membrane or a water soluble physiologically acceptable
salt thereof having the ability to pass through a cell
membrane, said assay compound having a leaving group
selected for cleavage by an enzyme to be analyzed and a
10   fluorogenic indicator group being selected for its
ability to have a non-fluorescent first state when
joined to the leaving group, and a fluorescent second
state excitable at a wavelength above 450 nm when the
leaving group is cleaved from the indicator group by the
15   enzyme, and at least one additive selected from the
group consisting of a buffer, an enzyme cofactor, an
enzyme modulator, an enzyme inhibitor, an enzyme
activator, a solubilizing component for said assay
reagent, and a retention component for said assay
20   reagent or products thereof, said assay reagent having
a fluorescence less than the auto-fluorescence of a
metabolically active cell.

        This invention also relates to a method to produce
an assay reagent for determining the activity of an
25   enzyme in a metabolically active whole cell, in which
the cell is contacted with the assay reagent. In a
broad aspect, the invention relates to a method to make
an assay compound for assaying the activity of an enzyme
inside a metabolically active whole cell, said assay
30   compound comprising an indicator group and a leaving
group, said leaving group being selected for cleavage by
said enzyme, comprising reacting a compound containing
a leaving group selected from the group consisting of
amino   acids,   peptides,   saccharides,   sulfates,
35   phosphates,   esters,   phosphate   esters,   nucleotides,
polynucleotides, nucleic acids, pyrimidines, purines,
nucleosides, lipids and mixtures thereof and a blocking

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group, with an agent to form an intermediate complex
containing a leaving group and a blocking group,
reacting the intermediate complex with a compound
containing an indicator group to form a reaction
5   product; separating the reaction product from side
reaction products, by-products and starting materials,
removing blocking groups from the reaction product to
obtain an assay compound having an indicator group and
leaving group, optionally reacting the intermediate
10   compound having an indicator group and leaving group
with an acid or base to form a physiologically
acceptable salt of said assay compound for assaying the
activity of an enzyme inside a metabolically active
whole cell, and purifying the assay compound or the
15   physiologically acceptable salt thereof.

More specifically, the present invention is further
related to a method for making an assay compound for
assaying the activity of an enzyme inside a
metabolically active whole cell, said assay compound
20   comprising an indicator group and a leaving group, said
leaving group being selected for cleavage by said
enzyme, comprising reacting a compound containing a
leaving group selected from the group consisting of
amino acids, peptides, saccharides, esters, nucleotides,
25   lipids and mixtures thereof, and a blocking group with
an agent to form an intermediate complex containing a
leaving group and a blocking group, reacting the
intermediate complex with a compound containing an
indicator group to form a reaction product, separating
30   the reaction product from side reaction products, by-
products and starting materials, removing the blocking
group from the reaction product to obtain an assay
compound having an indicator group and leaving group,
and purifying the assay compound.

35   In another embodiment, the present invention is
further related to a method for making an assay compound
in a salt form for assaying the activity of an enzyme

inside a metabolically active whole cell, said assay
compound comprising an indicator group and a leaving
group, said leaving group being selected for cleavage by
said enzyme, comprising reacting a compound containing

5   a leaving group selected from the group consisting of
amino acids, peptides, phosphates, sulfates, esters,
nucleotides and mixtures thereof, and a blocking group
with an agent to form an intermediate complex containing
a leaving group and a blocking group, reacting the

10   intermediate complex with a compound containing an
indicator group to form a reaction product, separating
the reaction product from side reaction products, by-
products and starting materials, removing the blocking
group from the reaction product to obtain an assay

15   compound having an indicator group and leaving group,
reacting the intermediate compound having an indicator
group and leaving group with an acid or a base to form
a physiologically acceptable salt of said assay compound
for assaying the activity of an enzyme inside a

20   metabolically active whole cell, and purifying the
physiologically acceptable salt of said assay compound.

The assay reagent has at least one assay compound
having an indicator group and a leaving group. The
leaving group is selected for cleavage by the enzyme to

25   be assayed. The indicator group is in a first state
when joined to the leaving group (e.g. the indicator is
non-fluorescent), and is in a second state when the
leaving group is cleaved from the indicator group by the
enzyme (e.g. the indicator group is fluorescent).

30   The present invention also relates to a method for
determining the activity of an endogenous enzyme in a
metabolically active whole cell, comprising contacting
a metabolically active whole cell with an assay reagent
under conditions which allow said assay reagent to pass

35   into said metabolically active whole cell, said assay
reagent having at least one assay compound having the
ability to pass through a cell membrane or a

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physiologically acceptable salt thereof having the ability to pass through a cell membrane, said assay compound comprising a fluorogenic indicator group and a leaving group, said leaving group being selected for

5 cleavage by said enzyme, said indicator group being in a non-fluorescent first state when joined to said leaving group, and being in a fluorescent second state excitable at a wavelength above 450 nm when said leaving group is cleaved from said indicator group by said

10 enzyme for a period of time sufficient for said assay reagent to be transferred into said cell and for said leaving group to be cleaved inside said cell from said indicator group by said enzyme, exposing said cell to light having a wavelength above 450 nm, and measuring

15 fluorescence of said cell.

The present invention also relates to a method for detecting an abnormality in the activity of an enzyme in a metabolically active whole cell, comprising (a) contacting a reference, metabolically active whole cell

20 having a normally functioning enzyme with a medium containing an assay reagent, said assay reagent having at least one water soluble assay compound having the ability to pass through a cell membrane or a water soluble physiologically acceptable salt thereof having

25 the ability to pass through a cell membrane, said assay compound comprising a fluorogenic indicator group and a leaving group, said leaving group being selected for cleavage by said enzyme, said indicator group being in a non-fluorescent first state when bonded to said

30 leaving group, and being in a fluorescent second state excitable at a wavelength above 450 nm when said leaving group is cleaved from said indicator group by said enzyme, for a period of time sufficient for said assay compound to be transferred into said cell and for said

35 leaving group to be cleaved inside said cell from said indicator group by said enzyme, (b) sensing for said fluorescent second state of said indicator group for the

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reference, metabolically active whole cell to produce reference results, (c) contacting a test, metabolically active whole cell with said medium for said period of time, (d) sensing for said fluorescent second state of

5   said indicator group for the test, metabolically active whole cell to produce test results, and (e) comparing the reference results of reference test, metabolically active whole cell in said step (b) with the test results obtained from said test metabolically active whole cell

10 in said step (d).

The present invention also relates to a method of performing an assay for detecting the presence of a disease comprising (a) contacting a test, metabolically active whole cell with an assay reagent, said assay

15 reagent containing at least one water soluble assay compound or water soluble physiologically acceptable salt thereof having a fluorogenic indicator group and a leaving group, said leaving group being selected for cleavage by a enzyme the activity of which changes with

20 the presence of the disease, said indicator group being in a non-fluorescent first state when bonded to said leaving group, and being in a fluorescent second state excitable at a wavelength above 450 nm when said leaving group is cleaved from said indicator group by said

25 enzyme for a period of time at least sufficient for said assay compound to be transferred into said cell and for said leaving group to be cleaved inside said cell from said indicator group by said enzyme, (b) sensing for said fluorescent second state of the indicator group for

30 the test, metabolically active whole cell to produce test results, and (c) comparing the test results of said test metabolically active whole cell with reference results obtained from at least one of a diseased reference cell and a non-diseased reference cell.

35 The present invention also relates to a method for detecting an abnormality in the activity of an enzyme in a   metabolically   active   whole   cell,   comprising

(a) contacting a plurality of reference, metabolically
active whole cells, each having at least one normally
functioning enzyme with a medium containing an assay
reagent, said assay reagent having at least one water

5   soluble assay compound having the ability to pass
through a cell membrane or a water soluble
physiologically acceptable salt thereof having the
ability to pass through a cell membrane, said assay
compound comprising a fluorogenic indicator group and a

10   leaving group, said leaving group being selected for
cleavage by one of said at least one normally
functioning enzymes, said indicator group being in a
non-fluorescent first state when bonded to said leaving
group, and being in a fluorescent second state excitable

15   at a wavelength above 450 nm when said leaving group is
cleaved from said indicator group by the one of said at
least one normally functioning enzyme, for a period of
time sufficient for said assay compound to be
transferred into each of said plurality of reference,

20   metabolically active whole cells for each of the at
least one normally functioning enzymes for each of said
plurality of reference, metabolically active whole cells
to produce a matrix of reference results and for said
leaving group to be cleaved inside of each of said

25   plurality of reference, metabolically active whole cells
from said indicator group by the one of said at least
one normally functioning enzymes, (b) sensing for said
fluorescent second state of said indicator group for
each of the at least one normally functioning enzymes

30   for each said plurality of reference, metabolically
active whole cells to produce a matrix of reference
results, (c) contacting a plurality of test,
metabolically active whole cells, each having at least
one normally functioning enzyme with said medium for

35   said period of time, (d) sensing for said fluorescent
second state of said indicator group for each of the at
least one normal functioning enzyme for each of said

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plurality of test, metabolically active whole cells to produce a matrix of test results, and (e) comparing the matrix of test results of said plurality of test, metabolically active whole cells in said step (d) with
5 the matrix of reference results obtained from said plurality of reference, metabolically active whole cells in said step (b).

The present invention also relates to a method of performing at least one or more assays for detecting the
10 presence of a disease comprising (a) contacting at least one or more test, metabolically active whole cells with one or more assay reagents, said assay reagent containing at least one water soluble assay compound or water soluble physiologically acceptable salt thereof
15 having a fluorogenic indicator group and a leaving group, said leaving group being selected for cleavage by one of said at least one normally functioning enzyme the activity of which changes with the presence of the disease, said indicator group being in a non-fluorescent
20 first state when bonded to said leaving group, and being in a fluorescent second state excitable at a wavelength above 450 nm when said leaving group is cleaved from said indicator group by the one of said at least one normally functioning enzyme for a period of time at
25 least sufficient for said assay compound to be transferred into said cell and for said leaving group to be cleaved inside said cell from said indicator group by said enzyme, (b) sensing for said fluorescent second state of the indicator group for the test, metabolically
30 active whole cell to produce a matrix of test results, and (c) comparing the matrix of test results of said test metabolically active whole cell with a matrix of reference results obtained from at least one of a diseased reference cell and a non-diseased reference
35 cell.

The cell is contacted with the assay compound for a period of time sufficient for the assay reagent to be

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transferred into the cell and for the leaving group to
be cleaved from the indicator group by the enzyme. The
assay compound is capable of or enabled to pass through
the membrane of the cell so that the enzyme, if present

5   and active, can cleave the leaving group thereby forming
the indicator compound which can be sensed from outside
the cell.

The cell is then sensed for the first state or
second state or both first and second states of the
10 indicator group.

BRIEF DESCRIPTION OF THE DRAWINGS

There are shown in the drawings embodiments which
are presently preferred, it being understood, however,
that the invention is not limited to the precise
15 instrumentalities and arrangements shown, wherein:

Figs. 1A, 1B, 1C and 1D are flow charts of four
assay protocols according to the invention;

Figs. 2A, 2B, 2C and 2D are charts illustrating the
use of salts to enhance specificity;
20   Figs. 3A, 3B, 3C and 3D are graphs illustrating the
use of inhibitors in the reagent formula;

Figs. 4A and 4B are photomicrographs of normal
Ficoll prepared lymphocytes and acute lympholytic Ficoll
prepared lymphocytes, respectively, which illustrate use
25 of an assay according to the invention to determine
immune competence and the difference in enzyme activity
between normal lymphocytes and acute lymphocytic
leukemia lymphocytes;

Figs. 5A and 5B are color photomicrographs which
30 illustrate use of an assay according to the invention to
provide an indication of leukemia;

Figs. 6A and 6B are color photomicrographs which
illustrate use of an assay to provide an indication of
sepsis;

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Fig. 7 is a color photomicrograph which illustrates the use of an assay to provide an indication of the metastatic potential of tumors;

Figs. 8A and 8B are color photomicrographs which
5   illustrate the use of assays to monitor drug treatment;

Fig. 9 is a color photomicrograph which illustrates the use of assays to provide an indication of macrophage activation;

Figs. 10A and 10B are graphs illustrating the
10   storage stability of a monopeptide-TFA salt derivative of rhodamine 110;

Figs. 11A, 11B, 11C, 11D, 11E and 11F are graphs illustrating the storage stability of TFA salts of dipeptide derivatives of rhodamine 110;

15   Figs. 12A, 12B, 12C and 12D are graphs illustrating the storage stability of acetate and tartrate salts of the Leu-Gly peptide derivative of rhodamine 110;

Figs. 13A and 13B are graphs illustrating the storage stability of free-amine peptide derivatives of
20   rhodamine 110;

Fig. 14A illustrates reducing a full covariance data matrix to a reduced covariance data matrix of strongly contributing factors, by eigenvector analysis;

Fig. 14B illustrates the prediction of disease
25   probabilities from the reduced covariance data matrix using Non-Negative Least Squares (NNLS) analysis;

Fig. 15 illustrates a full covariance data matrix being fed to a Neural Network to predict disease probabilities;

30   Fig. 16 illustrates a comparison of disease probabilities by performing 1) NNLS analysis on the full covariance data matrix, 2) NNLS analysis on a reduced covariance data matrix defined by eigenvectors 1 and 2, including 11 substrates, 3) NNLS analysis on a reduced
35   covariance data matrix defined by eigenvector 1 above, including 6 substrates, and 4) squared deviation from the mean analysis on 6 substrates;

Figs. 17A-17C are graphs of the ratio of a disease to the mean of the normal of all patients with the disease; and

Figs. 18A-18F illustrate a progression of disease during treatment and monitoring a return to normalcy and Fig. 18G is a summary of the data illustrated in Figs. 18A-18F.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Assay Compound

According to the present invention, an assay reagent is manufactured for determining the activity of an enzyme in a metabolically active whole cell. The assay reagent must be compatible with the cell such that the cell will remain metabolically active for at least the duration of the assay.

The assay reagent comprises at least one assay compound which is capable of passing through the cell wall. The assay compound must be small enough that it can be transmitted into the cell. An assay compound having a molecular weight of less than about 5,000 is presently preferred.

The assay compound contains a leaving group and an indicator group. The leaving group is selected for cleavage by the enzyme to be analyzed. The indicator group is selected for its ability to have a first state when joined to the leaving group, and a second state when the leaving group is cleaved from the indicator group by the enzyme. The indicator group is preferably excitable (caused to fluoresce) at a wavelength about the visible range, for example, at wavelength between about 450 to 500 nanometers (nm). The indicator group will usually emit in the range of about 480 to 620 nm, preferably 500 to 600 nm and more preferably 500 to 550 nm. Auto-fluorescence of the cell is most prevalent below about 500 nm.

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Indicator groups

The indicator group is preferably derived from fluorogenic and chemiluminescent compounds. The indicator group should be quenched when joined to the leaving group. The term quenched means that the indicator group has almost no fluorescence or chemiluminescence when joined to the leaving group. When the leaving group is separated from the indicator group, the resulting indicator compound will have a fluorescence.

Suitable fluorogenic indicator compounds include xanthine compounds. Preferably, the indicator compounds are rhodamine 110; rhodol; and fluorescein. These compounds have the following structures:

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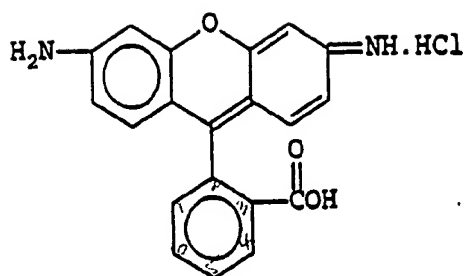
RHODAMINE 110
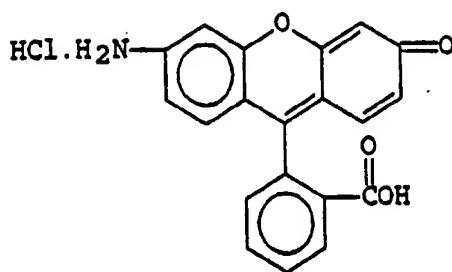
RHODOL
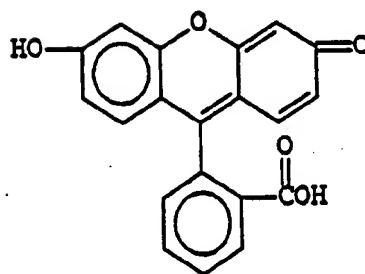
FLUORESCEIN

In addition, derivatives of these compounds which have the 4' or 5' carbon protected are acceptable indicator compounds. Preferred examples of the derivative compounds include 4'(5')thiofluorescein,

5 4'(5')-aminofluorescein, 4'(5')-carboxyfluorescein, 4'(5')-chlorofluorescein, 4'(5')-methylfluorescein, 4'(5')-sulfofluorescein, 4'(5')-aminorhodol, 4'(5')-carboxyrhodol, 4'(5')-chlororhodol, 4'(5')-methylrhodol, 4'(5')-sulforhodol; 4'(5')-aminorhodamine 110, 4'(5')-

10 carboxyrhodamine 110, 4'(5')-chlororhodamine 110, 4'(5')-methylrhodamine 110, 4'(5')-sulforhodamine 110 and 4'(5')thiorhodamine 110. "4'(5')" means that at the 4' or 5' position the hydrogen atom on the carbon atom is substituted with a specific organic group or groups

15 as previously listed.

Leaving groups

The leaving group is selected according to the enzyme that is to be assayed. The leaving group will have utility for assaying many kinds of cellular

20 enzymes, including proteases, glycosidases, glucosidases, carbohydrases, phosphodiesterases, phosphatases, sulfatases, thioesterases, pyrophosphatases, lipases, esterases, nucleotidases and nucleosidases. For the purposes of this disclosure the

25 term carbohydrases includes all enzymes which will hydrolyze a carbohydrate. Enzymes which do not recognize and cleave a leaving group, such as dehydrogenases and kinases, are not suitable for assays according to the invention. The enzymes to be measured

30 can be those which are present in various cell preparations, enzymes found in cytosols, cell surface enzymes, cytoplasmic enzymes and cell nucleus (nuclear) enzymes. However, as will be discussed herein, the assay compounds are particularly useful for detecting

35 intracellular enzymes in living cells.

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The leaving group is selected from amino acids, peptides, saccharides, sulfates, phosphates, esters, phosphate esters, nucleotides, polynucleotides, nucleic acids, pyrimidines, purines, nucleosides, lipids and
5   mixtures thereof. For example, a peptide and a lipid leaving group can be separately attached to a single assay compound such as rhodamine 110.

Other leaving groups suitable for the enzyme to be assayed can be determined empirically or obtained from
10 the literature. See, for example, Mentlein, R., Staves, R., Rix-Matzen, H. and Tinneberg, H.R., "Influence of Pregnancy on Dipeptidyl Peptidase IV Activity (CD26 Leukocyte Differentiation Antigen) of Circulating Lymphocytes", Eur. J. Clin. Chem. Clin. Biochem., 29,
15 477-480 (1991); Schön, E., Jahn, S., Kiessig, S., Demuth, H., Neubert, K., Barth, A., Von Baehr, R. and Ansorge, S., Eur. J. Immunol., 17, 1821-1826 (1987); Ferrer-Lopez, P., Renesto, P., Prevost, M., Gounon, P. and Chignard, M., "Heparin Inhibits Neutrophil-Induced
20 Platelet Activation Via Cathepsin", J. Lab Clin. Med. 119(3), 231-239 (1992); and Royer, G. and Andrews, J., "Immobilized Derivatives of Leucine Aminopeptidase and Aminopeptidase M.", The J. of Biological Chemistry, 248(5), 1807-1812 (1973). These references are hereby
25 incorporated by reference in their entirety. Various leaving groups are shown in Table 1.

TABLE 1

ENZYME	SUBSTRATES	SUB. CONC. (mM)	BUFF-ER	pH[1] RANGE (exper-iment)	pH[2] fit	COFAC-TOR	MODU-LATER	INHIBITOR	mOSM	TIME (MIN)	IONIC STRG (μ)	FUNCTION OR INDICATION	CD
AminoPept A	(H-L-Asp)$_2$-Rho 110-2TFA	4.8	Hanks	7.5	7.0	1-5mM CaCl$_2$		10 mM CaCl$_2$ Amistatin I	280-310	5	.1530		
	(H-L-Glu)$_2$-Rho 110-2TFA		Hanks		7.0				280-310	3			
AminoPept B	(H-L-Arg)$_2$-Rho 110-4TFA	3.2	Hanks	8.0 ±.2	7.5	NaCl 137 mM			280-310	3			
	(H-L-Cys)$_2$-Rho 110-2TFA		Hanks		7.5	1mM DTT			280-310	3			
AminoPept M	(H-L-Ala)$_2$-Rho 110-2TFA	6.4	Hanks	7.0	7.5			10^{-4}M Bestatin	280-310	5	0.1530	Leukemia	CD13
	(H-L-Ala)$_2$-4'chloro-Rho 110-2TFA	6.4	Hanks	7.0	7.5			10^{-4}M Bestatin	280-310	5	0.1530	Leukemia	CD13

etc.

ENZYME	SUBSTRATES	SUB. CONC. (mM)	BUFF-ER	pH¹ RANGE (exper-iment)	pH 2 fit	COFAC-TOR	MODU-LATER	INHIBITOR	mOSM	TIME (MIN)	IONIC STRG (μ)	FUNCTION OR INDICATION	CD
	H-L-Leu Rhodol-TFA	2.4-3.2	Hanks	5.0-7.0	7.5			1.5 mM 1,10-Phenanthro-line	280-310	1	0.1494		
	(H-L-Leu)₂ 4'chloro-Rho 110 -2TFA	2.4-3.2	Hanks	5.0-7.0	7.5				280-310	1	0.1494		
	(H-L-Leu)₂ Rho 110-2TFA	2.4-3.2	Hanks	5.0-7.0	7.5				280-310	1	0.1494		CD13
	(H-L-Met)₂ Rho 110-2TFA		Hanks		7.5			56 mM 2,2 Dipyridyl t	280-310	5			CD13
	(H-Gly)₂ Rho 110-2TFA	6	Hanks	7.0-7.5	7.5		1mM DTE		280-310	5	0.1526		CD13
	(H-Gly)₂ 4'chloro-Rho 110 -2TFA	6	Hanks	7.0-7.5	7.5		1mM DTE		280-310	5	0.1526		CD13
AminoPept M (cont.)	(H-L-Pro)₂ Rho 110-2TFA	6	Hanks		7.5		1mM DTE		280-310	5	0.1526		CD13
	(H-L-Lys)₂ Rho 110-4TFA	2.4	Hanks	7.5 ±.2	7.5				280-310	5	0.1490		CD13

ENZYME	SUBSTRATES	SUB. CONC. (mM)	BUFF-ER	pH[1] RANGE (exper-iment)	pH[2] lit	COFAC-TOR	MODU-LATER	INHIBITOR	mOSM	TIME (MIN)	IONIC STRG (μ)	FUNCTION OR INDICATION	CD
AminoPept N	(H-L-Lys)$_2$ Rho 110·4TFA	2.4	Hanks	5.5 ±.2	7.5				280-310	5	0.1490		
	(H Gly)$_2$ Rho 110·2TFA	6.4	Hanks	5.5-6.0	7.5				280-310	5			
	(H-L-Ser)$_2$ Rho 110·2TFA	2.4	Hanks	5.0-6.5	7.5				280-310	5	0.1506		
Neg Pro Control	(H-L-Pro)$_2$ Rho 110·2TFA	6	Hanks		7.5				280-310	5	0.1526		
DPP I	(H-L-Pro-Arg)$_2$ Rho 110·4TFA	6	MES	5.0-6.5	6.5		1mM DTT		280-310	10			
	(H Gly-Arg)$_2$ Rho 110·4TFA		MES	5.0-6.5	6.5		1mM DTT		280-310	10			
DPP II	(H-L-Lys-Ala)$_2$ Rho 110·4TFA	2.0	MES	6.5 ±.5	8.5	MgCl^2 Zn	DTE	Bestatin	280-310	10	0.1019		
	(H-L-Lys-Ala)$_2$ Rho 110-Salts·4TFA	2.0	Mes	6.5 ±.5	6.5	MgCl^2 Zn	DTE	Bestatin	280-310	10	0.1019		
	(H-L-Lys-Pro)$_2$ Rho 110·4TFA		MES	5.5	6.5	MgCl^2 Zn	DTE	Bestatin	280-310	10			

ENZYME	SUBSTRATES	SUB. CONC. (mM)	BUFFER	pH[1] RANGE (experiment)	pH[2] lit	COFAC-TOR	MODU-LATER	INHIBITOR	mOSM	TIME (MIN)	IONIC STRG (μ)	FUNCTION OR INDICATION	CD
DPP IV	(H-L-Ala-Pro)₂ Rho 110-2TFA		MES	5.5	6.5				280-310	10			
	(H-L-Lys-Ala-Lys-Ala)₂ Rho 110-6TFA	3.2	MES		6.5	$MgCl^2$ Zn	DTE	Bestatin	280-310	10	0.1228		
	(H-L-Ala-Pro)₂ Rho 110-2TFA				8.7				280-310	10			CD26
	(H-Gly-Pro)₂ Rho-2TFA	2.4	Gly-NaOH	7.5 ±.5	8.7			Gly-Pro 1.8mM	280-310	10	0.1449		CD26
	(H-L-Lys-Pro)₂ Rho 110-4TFA				8.7				280-310	10			CD26
	(H-L-Ala-Ala)₂ Rho 110-2TFA	4.0	Gly-NaOH		8.7		1mM DTE	3.6mM Ala-Ala	280-310	10	0.1449		CD26
	(H-Ala-Ala)₂ Rho 110-2TFA			8.7 ±.2	8.7		1mM DTE		280-310	10			
	(Z-Ala-Ala)₂ Rho 110		Gly-NaOH	7.0-8.5	8.0		1mM DTE		280-310	10			

ENZYME	SUBSTRATES	SUB. CONC. (mM)	BUFF-ER	pH¹ RANGE (experi-ment)	pH 2 lit	COFAC-TOR	MODU-LATER	INHIBITOR	mOSM	TIME (MIN)	IONIC STRG (µ)	FUNCTION OR INDICATION	CD
TriPeptidyl-Pept	(H-L-Ala-Ala-Ala-Ala)₂ Rho 110 -2TFA		Gly-NaOH		8.7		1mM DTE		280-310	10			CD26
	(H-L-Ala-Ala-Arg)₂ Rho 110 -4TFA		Gly-NaOH		8.7				280-310	10			
Cathepsin B	(H-L-Gln-Ser)₂ Rho 110 -2TFA	3.6	MES	6.5-7.5	6.5		1mM DTE	13mM-22mM Leupepsin I	280-310	10	0.1055	Breast Cancer	
	(H-L-Gln-Ser)₂ Rho 110 -2TFA		MES	5.5 ±.2	5.5				280-310	10			
	(H-L-Val-Ser)₂ Rho 110 -2TFA	4.0	MES	6.5-7.5	6.5			13mM-22mM Leupepsin I Cystatin C	280-310	10	0.1059	Tumor Growth	
Cathepsin B (cont.)	(H-L-Leu-Gly)₂ Rho 110 -2Tartrate	1.6-2.0	MES	6.0-6.5	6.5			13mM-22mM Leupepsin I Cysteine 2mM	280-310	10	0.1037	Progression	
	(H-L-Leu-Gly)₂ Rho 110 -2Acetate		MES	5.0-6.0	5.5				280-310	10			

ENZYME	SUBSTRATES	SUB. CONC. (mM)	BUFF-ER	pH1 RANGE (exper-iment)	pH 2 lit	COFAC-TOR	MODU-LATER	INHIBITOR	mOSM	TIME (MIN)	IONIC STRG (μ)	FUNCTION OR INDICATION	CD
	6H-I-Val-Lys]2 Rho 110-4TFA	1.6	MES		6.5		1mM DTE	13mM-22mM Leupeptin I	280-310	10	0.1072	Lung Tumor	
	6H-I-Leu-Leu-Arg]2 Rho 110 4TFA	6.4	MES		6.5			13mM-22mM Leupeptin I	280-310	10	0.1232	Leukemia	
	6H-I-Leu-Gly-Leu-Gly]2 Rho 110-2TFA	6.4	MES		6.5			13mM-22mM Leupeptin I	280-310	10	0.1083		
	6H-I-Val-Lys-Val-Lys]2 Rho 110 6TFA		MES		6.5		1mM DTE	Leupeptin I 1mM EDTA	280-310	10			
	6H-I-Ala-Arg]2 Rho 110 6TFA								280-310	10			
	6H-I-Lys-Arg]2 Rho110 6TFA							13mM-22mM Leupeptin	280-310	10		Gastric Cancer	
Cathepsin B1	6H-I-Leu-Leu-Arg]2 Rho 110 4TFA	6.4	MES	7.0 ±.5	6.5			13mM-22mM Leupeptin	280-310	10	0.1232	Smokers	

ENZYME	SUBSTRATES	SUB. CONC. (mM)	BUFFER	pH¹ RANGE (experiment)	pH² fit	COFAC-TOR	MODU-LATER	INHIBITOR	mOSM	TIME (MIN)	IONIC STRG (μ)	FUNCTION OR INDICATION	CD
	(H-L-Ala-Arg-Arg)₂ Rho 110 6TFA		MES		6.5			13mM-22mM Leupeptin					
	(Z-Ala-Gly)₂ Rho 110		Gly	7.5 ±.5	7.5				280-310	10			
	(H-L-Ala-Gly)₂ Rho 110 -2Acetate	2.4	Gly	8.0-8.5	8.7				280-310	10	0.1473		
Cathepsin C	(H-L-Thr-Pro)₂ Rho 110 4TFA	2.4	Gly	7.5-9.0	8.7				280-310	10	0.1473		
	(Z-Thr-Pro)₂ Rho 110			6.0-9.0	7.5				280-310	10			
	(H-L Pro-Arg)₂ Rho 110 4TFA		Gly		8.7				280-310	10			
Cathepsin D	(H Gly-Leu)₂ Rho 110 2TFA	1.2	MES	5.0 ±.5	6.5			10mM Pepstatin II	280-310	10	0.1031	Breast Cancer	

ENZYME	SUBSTRATES	SUB. CONC. (mM)	BUFF-ER	pH[1] RANGE (exper-iment)	pH[2] lit	COFAC-TOR	MODU-LATER	INHIBITOR	mOSM	TIME (MIN)	IONIC STRG (μ)	FUNCTION OR INDICATION	CD
	βH-L-Thr-Pro₂ Rho 110-2TFA	2.4	MES	5.0 ±.2	6.5				280-310	10	0.1043	MS	Liver Dis-ease
	βH-Gly-Pro-Leu-Gly-Pro₂ Rho 110-2TFA	3.2	MES	7.0 ±.5	6.5				280-310	10	0.1051	Leuk (ALL)	CD10
Neutral Endo Peptidase	βH-Gly-Phe-Gly-Ala₂ Rho 110-2TFA						Zinc ◿		280-310	10			(CAL-LA)
	βH-L-Arg-Gly-Glu-Ser₂ Rho 110-4TFA								280-310	10			
EndoPept I	βH-L-Arg₂ Rho 110-4TFA		.1M Tris Hcl		7.5				280-310	10			
	βH-L-Glu-Gly-Arg₂ Rho 110 4TFA								280-310	10			
EndoPept II	βH-L-Arg-Arg₂ Rho 110-6TFA		PO4		7.0		1mM DTE		280-310	10			

ENZYME	SUBSTRATES	SUB. CONC. (mM)	BUFF-ER	pH[1] RANGE (exper-iment)	pH 2 lit	COFAC-TOR	MODU-LATER	INHIBITOR	mOSM	TIME (MIN)	IONIC STRG (µf)	FUNCTION OR INDICATION	CD
	(H-L-Ala-Arg-Arg)₂ Rho 110 6TFA						1mM EDTA		280-310	10			
Membrane Assoc. EndoPept I	(H-Gly-Ala-Ala-Ala)₂ Rho 110 2TFA								280-310	10			
	(H-L-Arg-Arg)₂ Rho 110 6TFA								280-310	10			
Membrane Assoc EndoPept II	(H-L-Ala-Arg-Arg)₂ Rho 110 6TFA								280-310	10			
Glutathione	(H-L-Glu-Cys-Gly)₂ Rho 110 2TFA								280-310	10			
Chymotrypsin	(H-Glu-Gly-Phe)₂ Rho 110 2TFA	.1M Tris			7.0			PMSF	280-310	10			

ENZYME	SUBSTRATES	SUB. CONC. (mM)	BUFFER	pH[1] RANGE (experiment)	pH[2] fit	COFACTOR	MODULATER	INHIBITOR	mOSM	TIME (MIN)	IONIC STRG (μ)	FUNCTION OR INDICATION	CD
Trypsin	(H-L-Arg)2 Rho 110-4TFA				7.0			Antipain	280-310	10			
	(H Gly-Gly-Arg)2 Rho 110 -4TFA							13mM- 22mM Leupeptin	280-310	10			
Ester Proteinase	(N-Acetyl MET)2 Rho 110	.1M PO4			6.5					10			
γ-GT	(γ-Glu)2 Rho 110-2TFA				7.0		1mM Gly-Gly		280-310	10			
	(H-L-Ala-Ala-Tyr)2 Rho 110 -2TFA							α-1- Antitrypsin	280-310	10			
Elastase	(H-L-Ala-Ala-Pro-Ala)2 Rho 110 -2TFA								280-310	10			
	(H-L-Ala-Ala)2 Rho 110 -2TFA								280-310	10			
	(H-L-Ala-Pro-Ala)2 Rho 110 -2TFA								280-310	10			

ENZYME	SUBSTRATES	SUB. CONC. (mM)	BUFF-ER	pH¹ RANGE (experiment)	pH² lit	COFAC-TOR	MODU-LATER	INHIBITOR	mOSM	TIME (MIN)	IONIC STRG (µ)	FUNCTION OR INDICATION	CD
Plasmin	βH-L-Ala-Phe-Lys)₂ Rho 110 4TFA							13mM-22mM Leupeptin I	280-310	10			
	βH-L-Glu-Lys-Lys)₂ Rho 110 6TFA								280-310	10			
	βH-L-Val-Leu-Lys)₂ Rho 110 4TFA								280-310	10			
Urokinase	βH-Gly-Gly-Arg)₂ Rho 110 4TFA								280-310	10			
	βH-Gly-Arg)₂ Rho 110 4TFA								280-310	10			
HIV Protease	βH-L-Lys-Ala-Arg-Val)₂ Rho 110 6TFA								280-310	10			
	βH-L-Lys-Ala-Arg-Val-Phe)₂ Rho 110 6TFA								280-310	10			
v-Thrompsin	βH-Val-Pro-Arg)₂ Rho 110 4TFA							PMSF	280-310	10			
Pancreatic	βH-L-Pro-Phe-Arg)₂ Rho 110 4TFA								280-310	10			

ENZYME	SUBSTRATES	SUB. CONC. (mM)	BUFF-ER	pH¹ RANGE (exper-iment)	pH 2 lit	COFAC-TOR	MODU-LATER	INHIBITOR	mOSM	TIME (MIN)	IONIC STRG (μ)	FUNCTION OR INDICATION	CD
Cathepsin L	(H-Phe-Arg)₂ Rho 110·4TFA							13mM-22mM Leupeptin	280-310	10		Breast Carcinoma growth cancer	
Cathepsin H	(H-L-Arg)₂ Rho 110·4TFA			8.0 ±.2					280-310	10		Breast Carcinoma	
Collagenase	(H-Gly-Pro-Leu-Gly-Pro)₂ Rho 110·2TFA		MES	5.5 ±.2	5.5	$MgCl^2$	DTE	Bestatin	280-310	10			

ENZYME	SUBSTRATES	SUB. CONC. (mM)	BUFFER	pH[1] RANGE (experiment)	pH[2] lit	COFAC-TOR	MODU-LATER	INHIBITOR	mOSM	TIME (MIN)	IONIC STRG (µ)	FUNCTION	CD
Neutral Esterase	Fl(palmitate)₂	.1	Hanks	7.5					280-310	10	.15		
Acidic Esterase		.1	Mes	6.5					280-310	10	.10		
Acid Phosphatase	Fl(phosphate)₂·2NH₄⁺	.2	Mes	5.0					280-310	5	.10		
Alkaline Phosphatase	Fl(phosphate)₂·2NH₄⁺	.2	Gly	8.7					280-310	5			
Tartrate Resistant Phosphatase	Fl(phosphate)₂·2NH₄⁺	.2	Tartrate Mes	5.2					280-310	5	.10	Hairy Cell Leukemia	
Acid Phosphatase	Rho 110(phosphate)₂	.1	Mes	5.0					280-310	5			
Alkaline Phosphatase	Rho 110(phosphate)₂	.1	Gly	8.7					280-310	5			
Tartrate Resistant Phosphatase	Rho 110(phosphate)₂	.1	Tartrate Mes	5.2					280-310	5			
Neutral Non-specific Esterase	Fluorescein (acetate)₂	.1	Hanks	7.5					280-310	3	.15	Monocytes, Megakaryo-cytes, Lympho-cytes	
Acidic Non-specific Esterase	Fluorescein (acetate)₂	.1	Mes	4.0-6.5					280-310	3	.10		

ENZYME	SUBSTRATES	SUB. CONC. (mM)	BUFFER	pH¹ RANGE (experiment)	pH² fit	COFAC-TOR	MODU-LATER	INHIBITOR	mOSM	TIME (MIN)	IONIC STRG (μ)	FUNCTION	CD
Neutral Esterase	FL(propionate)₂	.1	Hanks	7.5					280-310	3	.15		
Acidic Esterase		.1	Mes	6.5					280-310	3	.10		
Neutral Chloroacetate Esterase	FL(chloroacetate)₂	.1	Hanks	7.5					280-310	3	.15	Immature Neutrophils & Mast cells	
Acidic Chloroacetic Esterase		.1	Mes	6.5					280-310	3	.10		
Neutral Esterase I	FL(butyrate)₂	.1	Hanks	7.5					280-310	3	.15	Monocytes &	
Acidic Esterase I		.1	Mes	6.5					280-310	3	.10	Megakaryo-cytes	
Neutral Esterase I	FL(chlorobutyrate)₂	.1	Hanks	7.5					280-310	3	.15		
Acidic Esterase I		.1	Mes	6.5					280-310	3	.10		
Neutral Esterase	FL(valerate)₂	.1	Hanks	7.5					280-310	3	.15		
Acidic Esterase		.1	Mes	6.5					280-310	3	.10		

ENZYME	SUBSTRATES	SUB. CONC. (mM)	BUFFER	pH¹ RANGE (experiment)	pH² fit	COFAC-TOR	MODU-LATER	INHIBITOR	mOSM	TIME (MIN)	IONIC STRG (μ)	FUNCTION	CD
Neutral Esterase	Fl.(hexanoate)₂	.1	Hanks	7.5					280-310	3	.15		
Acidic Esterase		.1	Mes	6.5					280-310	3	.10		
Neutral Esterase	Fl.(heptanoate)₂	.1	Hanks	7.5					280-310	3	.15		
Acidic Esterase		.1	Mes	6.5					280-310	3	.10		
Glycopyranosidase	(Acetyl-α-D-glucopyranosyl)₂ Rho 110	.24	Mes	6.8					280-310	10			
Glucuronidase	(β-D-glucuronide)₂ Rho 110	.24	Mes	5.0					280-310	10		Leukemia	
Galactopyranosidase	(β-D-Galactopyranoside)₂ Rho 110	.24	Hanks	7.5					280-310	10			
Tyrosine Phosphatase	(H-L-Tyrosine Phosphate)₂ Rho 110-2TFA	.1	Hanks	6.5					280-310	5		Cell Cycle Cell Division	
Serine Phosphatase	(H-L-Serine Phosphate)₂ Rho 110-2TFA	.1	Hanks	6.5					280-310	5		Cell Cycle Cell Division	

ENZYME	SUBSTRATES	SUB. CONC. (mM)	BUFFER	pH¹ RANGE (experiment)	pH² lit	COFAC-TOR	MODU-LATER	INHIBITOR	mOSM	TIME (MIN)	IONIC STRG (μ)	FUNCTION	CD
Threonine Phosphatase	[³H-Threonine Phosphate]₂ Rhe 110-2TFA	.1	Hanks	6.5					280-310	5		Cell Cycle Cell Division	
Neutral Esterase II	[³H-Acetyl-L-Ala]₂-Fl	.1	Hanks	7.5					280-310	5		Monocytic Leukemias	45 RO
Acidic Esterase II		.1	Mes	6.5					280-310				
Adenosine Deaminase	(Adenosine)₂ Rhe 110-2TFA	.1	Mes	6.0					280-310	10		AIDS	
Thymidine Deaminase	(Thymidine)₂ Rhe 110	.1	Mes	6.0					280-310	10			
Cytosine Deaminase	(Cytosine)₂ Rhe 110-2TFA	.1	Mes	6.0					280-310	10			
Guanine Deaminase	(Guanine)₂ Rhe 110-2TFA	.1	Mes	6.0					280-310	10			
5'Nucleotidase	(Adenosine Monophosphate)₂ Rhe 110-2TFA	.1	Hanks	7.5 - 9.0					280-310	10		Pap Smear	

ENZYME	SUBSTRATES	SUB. CONC. (mM)	BUFFER	pH¹ RANGE (exper-iment)	pH² lit	COFAC-TOR	MODU-LATER	INHIBITOR	mOSM	TIME (MIN)	IONIC STRG (μ)	FUNCTION	CD
Adenine Monophosphate Deaminase	Rho 110(AMP)₂·4NH₄⁺	.1	Mes	6.0					280-310	10		AIDS	
Angiotensin Converting Enzyme	(Hippuryl-His-Leu)₂ Rho 110	.1	HEPES	8.0					280-310	10		CALLA	
Cholinesterase	F(Choline)₂	.1	Hanks	8.0		Zn⁺⁺			280-310	10			
Cholinesterase	F(Butyryl Thiocholine)₂	.1	Hanks	8.0		Zn⁺⁺			280-310	10			
Acetyl Cholinesterase	F(Acetyl-Choline)₂	.1	Hanks	8.0		Zn⁺⁺			280-310	10			
Nucleosidase	(Adenine)₂ Rho 110-2TFA	.1	Hanks	7.4					280-310	10		Pap Smear	
Lipase	(Saturated Hydrocarbon)₂ Rho 110	.1											
Lipase	(Unsaturated Hydrocarbon)₂ Rho 110	.1											
Lipase	(Triacetin)₂ Rho 110	.1	Hanks	7.7					280-310	10			
Phospholipase	Rho 110 (Phosphatidyl-choline)₂·2TFA	.1	Hanks	7.0							.15		

ENZYME	SUBSTRATES	SUB. CONC. (mM)	BUFFER	pH[3] RANGE (exper-iment)	pH[2] lit	COFAC-TOR	MODU-LATER	INHIBITOR	mOSM	TIME (MIN)	IONIC STRG (µ)	FUNCTION	CD
Phospholipase C	Rho 110 (Phosphatidyl-inositol)$_2$	.1	Hanks	7.0					280-310	10	.15		
Phospholipase D	(Phosphatidyl-choline)$_2$ Rho 110-2TFA	.1	Hanks	7.0					280-310	10	.15		
Phospholipase A	(Phosphatidyl-choline)$_2$ Rho 110-2TFA	.1	Hanks	7.0					280-310	10	.15		
	(H-tyrosine)$_2$ Rho 110-2TFA												

1. Range determined experimentally with cells
2. pH from scientific literature using cytosol

Preferred peptide leaving groups that react with cellular enzymes are included in Table 1. As examples, the enzymes glutamyltranspeptidase reacts with gamma-glutamyl amino acid peptide giving gamma glutamic acid; 5 trypsin cleaves the peptide at the arginine residue; aminopeptidase-M hydrolyzes the peptide at the aliphatic amino acid residue; and chymotrypsin cleaves the peptide at the phenylalanine residue.

It has been discovered that when the leaving group 10 is a salt complex, it will significantly improve the transmission of the assay compound into the cell. The selection of an appropriate salt complex requires a consideration of the compatibility with the cell, solubility in the aqueous media, and cleavage by the 15 enzyme. Particular care is required in the selection of the peptide salt since even isoenzymes have been found to be specific in their recognition of particular salts.

Leaving groups for saccharidases are preferably prepared by the synthesis of monosaccharides, 20 oligosaccharides or polysaccharides comprising between one and about ten sugar residues of the D-configuration. Examples of useful sugars are monosaccharides-pentoses; ribose; deoxyribose; hexose: glucose, dextrose, galactose; oligosaccharides-sucrose, lactose, maltose 25 and polysaccharides like glycogen and starch.

The sugar can be an alpha or beta configuration containing from 3 to 7 and preferably 5 to 6 carbon atoms. Analogs of these sugars can also be suitable for the invention. Preferably, the D-configuration of the 30 monosaccharide or disaccharide is utilized. The monosaccharide or disaccharide can be natural or synthetic in origin.

Leaving groups for nucleases, nucleotidases, and nucleosidases are preferably prepared by the synthesis 35 of nucleic acids, purines, pyrimidines, pentose sugars (i.e., ribose and deoxyribose) and phosphate ester. Examples are adenine, guanine, cytosine, uracil and

thymine. Leaving groups for restriction enzymes would include polynucleotides.

The nucleic acids contain a purine or pyrimidine attached to a pentose sugar at the 1-carbon to N-9 purine or N-1 pyrimidine. A phosphate ester is attached to the pentose sugar at the 5' position. Analogs of these building blocks can also be used.

Leaving groups for lipases are preferably prepared by the synthesis of simple lipids, compound lipids or derived lipids. Simple lipids can be esters of fatty acids, triglycerides, cholesterol esters and vitamin A and D esters. Compound lipids can be phospholipids, glycolipids (cerebrosides), sulfolipids, lipoproteins and lipopolysaccharides. Derived lipids can be saturated and unsaturated fatty acids and mono or diglycerides. Analogs of these lipids can also be used.

Examples of lipids are: triglycerides - triolein, fatty acids - linoleic, linolenic and arachidonic; sterols - testosterone, progesterone, cholesterol; phospholipids - phosphatidic acid, lecithin, cephalin (phosphatidyl ethanolamine) sphingomyleins; glycolipids - cerebosides, gangliosides.

Leaving groups for esterases are preferably prepared by the synthesis of carboxylic acids comprising between 2 and 30 carbon atoms. The carboxylic acids can be saturated or unsaturated. The carboxylic acid preferably contains 2 to 24 carbons and more preferably 4 to 24 carbon atoms. Analogs of theses carboxylic acids can also be used. The carboxylic acids can be natural or synthetic in origin. Examples are butyric, caproic, palmitic, stearic, oleic, linoleic and linolenic.

Leaving groups for phosphatases are preferably prepared by the synthesis of phosphates, phosphatidic acids, phospholipids and phosphoproteins. Analogs of these compounds can also be used. Examples are ATP, ADP, AMP and cyclic AMP (c-AMP).

Leaving groups for peptidases are preferably prepared by the synthesis of peptides comprising between one and about ten amino acid residues of the L-configuration. Typically, it has been found that the synthesis of peptides having more than about six amino acids produces a low yield. However, where the yield is acceptable, peptides of greater length can be employed.

The amino acids preferably contain 2-10 and preferably 2-8 carbon atoms. Analogs of these amino acids can also be suitable for the invention. If the amino acids are chiral compounds, then they can be present in the D- or L- form or also as a racemate. Preferably, the L- configuration of the amino acid is utilized. The amino acids of the oligopeptide can be natural and/or of synthetic origin. Amino acids of natural origin, such as occur in proteins and peptide antibiotics, are preferred. Synthetic amino acids can also be used, such as pipecolic acid, cyclohexylalanine, phenylglycine, α-aminocyclohexylcarboxylic acid, hexahydrotyrosine, norleucine, or ethionine.

Protecting (Blocking) Groups

Protecting groups are preferably employed when synthesizing the leaving group to prevent undesired side reactions of the leaving group during synthesis of the assay compound. N-terminal protecting groups and polar organic protecting groups on the other portion of the amino acid molecule are used to prevent undesired side reactions of the amino acids during syntheses of the peptides. The protecting groups, also known as blocking groups, are removed prior to the assay, unless the presence of a particular blocking group or groups is found not to interfere with the assay.

The N-terminal protecting groups include an arylcarbonyl, alkylcarbonyl, alkoxycarbonyl, aryloxycarbonyl, aralkoxycarbonyl, arylsulfonyl, alkylsulfonyl, or other equivalents known to those

skilled in the art of peptide syntheses. The polar
organic protective groups include hydroxyl, guanidinyl,
sulfhydryl and carboxyl or other equivalents known to
those skilled in the art of peptide syntheses. Gross
5   and Meienhofer, eds., The Peptide, 3(3-81) (Academic
Press, New York, 1981), describe numerous suitable amine
protecting groups.

Preferred examples of the N-terminal blocking
groups include formyl, acetyl, trifluoroacetyl,
10 benzyloxycarbonyl, phthaloyl, benzoyl, acetoacetyl,
chloroacetyl, phenoxycarbonyl, carbobenzoxy, substituted
benzyloxycarbonyl, tertiarybutyloxycarbonyl,
isopropyloxycarbonyl, allyloxycarbonyl, phthaloyl,
benzoyl, acetoacetyl, chloroacetyl, phenoxycarbonyl,
15 methoxysuccinyl, succinyl, 2,4-dinitrophenol, dansyl, p-
methoxybenzenesulfonyl, and phenylthio.

Preparation of Intermediate Complex

A compound containing a blocking group and a
leaving group such as an amino acid is reacted with an
20 agent to form an active intermediate complex. The
leaving group is selected based on the leaving group
desired in the final assay compound. Suitable agents
are known to those skilled in the art of peptide
chemistry. Examples of suitable agents include
25 carbodiimides, (preferably 1-ethyl-3-(3'-
dimethylaminopropylcarbodiimide hydrochloride) and
benzotriazolyl-N-oxy-tris(dimethylamino)phosphonium
hexafluorophosphate (BOP reagent) and 1-
hydroxybenzotriazole (HOBT reagent). The reagents are
30 typically stirred in a flask at room temperature. The
chemical structure of the intermediate complex is
presently unknown. The presence of the complex can be
confirmed by thin layer chromatography.

Preparation of Reaction Product

The intermediate complex is further reacted with a compound containing an indicator group (indicator compound) to form a reaction product. As appreciated by

5   those skilled in the art of peptide chemistry, the indicator compound is dissolved in a solvent to facilitate the reaction with the intermediate complex. The reagents are typically stirred in a flask at room temperature for a time sufficient to form a reaction

10 product. The reaction product can be confirmed by developing a thin layer chromatography (TLC) plate in an organic solvent. The reaction product should be a non-fluorescent compound. When the indicator group is rhodamine 110, rhodol or a derivative, the presence of

15 the reaction product is confirmed by contacting the reaction product with an acidic solution, such as hydrochloric acid, which cleaves the leaving group thereby forming a colored product. When the indicator group is fluorescein or a derivative, the presence of

20 the reaction product is confirmed by contacting the reaction product with a basic solution, such as sodium hydroxide, which cleaves the leaving group thereby forming a colored product. If only one spot on the TLC plate gives a positive test and there are no trace

25 amounts of fluorescence or its derivatives, the reaction product is of acceptable purity for this stage of the process.

Purification of Reaction Product

The reaction product is then separated from other

30 side reaction products, by-products and starting materials in the following manner. Preferably, the reaction product is concentrated to an oil under reduced pressure so as to remove volatile solvents that might be present. The reaction product oil is then redissolved

35 in a minimum of an organic solvent, preferably chloroform, methylene chloride, and further separated

from the other side reaction products, by-products and
starting materials by normal phase preparative high
pressure liquid chromatography (HPLC). Other
conventional methods of separation can be employed.
5   Separation of the reaction product is verified by TLC,
as previously described, and analytical reverse phase
HPLC. The reverse phase HPLC will depict the presence
of one major band of reaction product.

The reaction product is separated from the other
10 side reaction products, by-products and starting
materials so that the reaction product can be further
processed by having the blocking groups removed. If the
reaction product is not sufficiently separated from the
other side reaction products, by-products and starting
15 materials, then a low yield of the assay compound
containing an indicator group and leaving group will be
obtained. Moreover, the quality of the separation will
have an effect on the amount of purification that will
be subsequently necessary to obtain an assay compound
20 for use in the metabolically active cell.

Removal of Blocking Group

The blocking group which is blocking (protecting)
the leaving group is then removed from the reaction
product to obtain an assay compound ("intermediate
25 compound" if a salt is to be formed) which contains an
indicator group and a leaving group. The reactions are
conducted to obtain a free amino acid xanthine
derivative by methods known to those skilled in the art.
When the blocking group on the indicator group comprises
30 benzyloxycarbonyl (CBZ), the blocking group is removed
by a catalytic reaction of the reaction product in an
organic solvent with hydrogen in the presence of
palladium or platinum. Further details of this process
are shown in Example 13. When the blocking group on the
35 indicator group comprises 9-fluorenylmethyloxycarbonyl
(FMOC), the blocking group is typically removed by the

reaction of the reaction product in a polar solvent with an organic base. Further details of this process are shown in Example 1.

To confirm that the blocking group has been removed
5   from the resulting intermediate compound, the intermediate compound is analyzed by analytical reverse phase HPLC. In addition, the resulting intermediate compound can be further confirmed by developing a thin layer chromatography plate in an organic solvent.

10   **Physiologically Acceptable Salt Formation**

This intermediate compound having an indicator group and leaving group is then reacted with an acid or a base to form an assay compound, which is a physiologically acceptable salt. It is important
15   according to the method of the invention that the assay compounds be physiologically acceptable to the cell. The selection of the acid or base has a material affect on whether the resulting assay compound will be physiologically acceptable to the cell. In addition, it
20   has been found that the selection of the acid affects the selectivity of the assay compound for the enzyme to be assayed.

It has been found that hydrogen bromide (HBr), even when buffered, kills cells. To confirm whether an acid
25   will be appropriate to use, a selected acid is used to make an assay compound. The assay compound is then tested with a metabolically active cell to determine if viability (Trypan Blue; propidium iodide-fluorescein diacetate [PI-FDA]) over the assay time period is
30   affected. Viability is confirmed with Trypan Blue or PI-FDA over a time period of 10 seconds to 30 minutes. If the viability of the cell sample at between one and three million cells/mL decreases by 10% then the salt of the compound is rejected and another salt of the assay
35   compound is synthesized.

Preferably the acid that is used to form the salt
is selected from the group consisting of hydrochloric,
sulfuric, nitric, maleic, acetic, trifluoroacetic,
tartaric acid, citric, succinic and p-toluenesulfonic
5   acid.   More preferably the acid is selected from the
group consisting of acetic, trifluoroacetic, tartaric
acid, and p-toluenesulfonic acid. Most preferably the
acid is trifluoroacetic. When a base is used, ammonia
or organic bases can be used. Most preferably, the base
10   is ammonia.

Purification of the Assay Compound

The assay compound is purified, preferably by
reverse phase HPLC. It is very important that the side
reaction products, by-products and starting materials
15   from the synthesis of the assay compound be removed
which would diminish the utility of the assay. Non-
physiologically acceptable impurities should be removed.
In addition, the background noise generated from
impurities should be less than the auto-fluorescence of
20   a metabolically active cell.

It has been found that when a leaving group is
present as an impurity, the leaving group can be an
inhibitor to enzyme activity. Still further, metal
impurities in any of the starting materials can poison
25   the enzymes, prevent hydrolysis of the assay compound
and interfere with the accuracy of the enzyme assay.

In addition, impurities will create background
fluorescence which will add to the natural fluorescence
of the cell to create a level of background noise which
30   can interfere with the detection of enzyme generated
fluorescence. Fluorescent impurities can be taken up by
the cell, and a rate measurement of fluorescence against
time will show a false rate of increasing fluorescence
that is due only to this cellular uptake of fluorescent
35   impurities. This is a particular problem if the assay
is conducted to determine the presence or absence of an

enzyme, since this impurity will indicate a rate of fluorescence which will falsely appear to be attributable to enzymatic activity.

5   The assay compound can be purified by techniques known in the art.   As shown in Example 1, the purification of rhodamine 110 substrate can be accomplished by reverse phase column chromatography.

In the case of the preparation of salts of peptide-rhodamine 110 compounds, a significant level of

10   impurities is created.   These impurities include free indicator compound, monosubstituted rhodamine 110, blocked amino acids and peptides.

The fluorescence impurities should be removed to a level that they do not obscure the baseline detection of

15   the enzyme in the cell.   The baseline detection can be established by analyzing log dilutions of an indicator group. Preferably the impurities should be removed so that the fluorescence of the impurities is less than the auto-fluorescence of the metabolically active cell.

20   Assays   for   peptidases   using   assay   compounds generate fluorescence generally in the range of 10^{-5} to 10^{-6} Molar free rhodamine 110.   Therefore, it is preferred that the free rhodamine 110 and blocked peptide impurities in the assay reagent should be

25   removed to a concentration of less than the fluorescence generated by about 1×10^{-6} M and more preferably less than the fluorescence generated by about 10^{-7} Molar free indicator group. This amounts to a 100,000 photon count using rhodamine 110 as a standard at 10^{-7}-10^{-8} M,

30   preferably 5×10^{-8} M in a 1 cm path length cuvette when measured   over   10   min.   on   a   photon   counting spectrofluorometer manufactured by the SLM Company of Chicago, Illinois. This corresponds to a use level on the flow cytometer where no cellular false positive can

35   be detected for a 10 minute period at the highest sensitivity setting.   In the case of the peptide-rhodamine 110 compounds, this has been found to require

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a concentration of impurities of less than one part per one hundred thousand, more preferably less than one part per five hundred thousand, most preferably less than one part per million. The presence of impurities causes a

5   decrease in the storage stability of the compound, resulting in an increased autohydrolysis which leads to increased background fluorescence. A compound should be free of impurities such that when the compound (or reagent containing the compound) is stored at 4°C for 30

10 days, preferably 90 days, more preferably 180 days, most preferably one year, the background fluorescence increases less than 10%, preferably less than 5%, most preferably less than 1% over these time periods, respectively. The purified compound or lyophilized

15 reagent are stored in a sealed container over dry nitrogen under atmospheric pressure. The starting point in time for measuring stability is usually immediately after purification of the assay compound is completed but it can be any time such as immediately after the

20 preparation of the assay reagent is completed.

Normal phase preparative HPLC procedures are presently preferred to separate peptide-indicator compound from the impurities. As is known in the art, solvents of varying polarity can be mixed in varying

25 concentrations in order to more effectively separate the peptide-indicator compound from the various impurities. Thin layer chromatography (TLC) can be utilized to test for the presence of the rhodamine 110 substrate in the eluate. This is done by placing a drop of the eluent on

30 the TLC plate, and then treating the spot with a suitable acid, such as HCl, to detect the presence of the rhodamine 110 substrate, which will turn bright yellow when treated with acid. Analytical reverse phase high pressure liquid chromatography is used to test the

35 peptide-indicator product for purity, as evidenced by a single sharp band in the absorption spectrum.

Preparation of Assay Reagent

The assay reagent must be compatible with the metabolically active cell. The assay reagent should have an osmolality of from about 250 milliosmoles to 350 milliosmoles, preferably from about 275 milliosmoles to 320 milliosmoles. Further, the assay reagent will have an ionic strength between about 0.1 to 0.3 μ. In addition, the pH of the assay reagent will be between about 4.0 and 9.5, preferably between about 5.0 and 8.0. The preferred pH for assay compounds for particular enzymes is included in Table 1.

It has been further found that the efficacy of an intracellular assay is substantially improved by the addition of one or more components in the assay reagent. Examples of improvements include a reduction of reaction time, increased selectivity for the targeted enzyme, reduction of competing enzyme reactions, increasing signal of enzyme reaction, increasing reactivity of the assayed enzyme relative to other non-targeted enzymes, increasing the retention time of the indicator group within the cell and other similar advantageous results.

Additional components include buffers, cofactors, modulators, inhibitors, activators for increasing activity of the target enzymes over other non-targeted enzymes, solubilizing components and retention components can be included in the assay reagent to improve the enzyme assay results. These components are physiologically acceptable to the metabolically active whole cell that is being assayed.

The chemical nature of the buffer is important to the reactivity of the assay compound with the cellular enzymes. For example, it has been found that Hanks solution is a better cellular buffer than cacodylic acid at 0.1 M concentration for amino peptidase. More specifically, by utilizing Hanks solution, at pH 7.5, it has been further found that the assay compound has a higher sensitivity for the targeted enzyme. In

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addition, the assay compound hydrolysis by the enzyme occurs at an increased rate of reaction. Although Hanks solution contains calcium chloride at a concentration of 1.26 mM, calcium chloride has been found in the case of

5   aminopeptidase to be inhibitory to the enzyme reaction with the assay compound, (H-L-Asp)$_2$ rhodamine 110, at concentrations of approximately 10 mM.

Buffer components that show no inhibitory effect to the cells can be used. Suitable buffer components are

10 N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES),   Hanks   balanced   salt   and   2-N-morpholinoethanesulfonic   acid   (MES),   tris-glycine, HEPES, glycine sodium hydroxide, and cacodylate. The preferred   buffer   components   are   MES   for   acidic

15 solutions, Hanks for neutral solutions, and glycine sodium hydroxide for basic solutions. Preferred buffers for particular enzymes are included in Table 1.   A metabolic energy source such as a sugar (glucose) can be added.

20   Cofactors are components not consumed in the enzymatic reactions, but are required to make the enzyme function. Suitable cofactors include metals such as Ca^{+2}, Zn^{+2}, Mg^{+2}, Fe^{+2} and Mn^{+2}. These cofactors can increase the selectivity of the enzyme for the leaving

25 group.   The cofactors can also be co-enzymes or vitamins. Preferred cofactors for particular enzymes are included in Table 1.

Modulators are components used to decrease the sensitivity of the enzyme for the leaving group. The

30 modulators speed up or slow down the activity of an enzyme by changing the active site. Therefore, enzyme activity can be down-regulated, as in negative feedback inhibition by the leaving (stimulated) group inhibiting the original enzyme. For example,   dithioerythritol

35 (DTE) at 1 mM decreases the sensitivity of the substituted rhodamine 110 substrates containing the amino acids Pro, Gly, Gln-Ser, Val-Lys-Val-Lys, Ala-Ala,

and Ala-Ala-Ala-Ala, but does not change the sensitivity
of the leaving group for the enzyme where the substrate
contains the amino acids Ala-Gly, and Leu-Gly.
Dithiothreitol (DTT) has also been found to be an
5   effective modulator. Preferred modulators for
particular enzymes are included in Table 1.

Inhibitors and poisons (or toxins) are components
that can be added to reduce the activity of non-targeted
enzymes that provide competing reactions for the leaving
10   group. Inhibitors are usually very selective for a
particular enzyme. For example, EDTA only works with
enzymes requiring Ca^{+2}, Mg^{+2} and some other metals.
Other examples of an inhibitor are bestatin, which
selectively inhibits aminopeptidase and leupeptin which
15   selectively inhibits cathepsin B. In addition, the
monopeptide reagent contains approximately 137.9 mM per
liter of sodium chloride. However, the addition of 7 mM
of sodium chloride to the dipeptide reagent has a
slightly inhibitory effect over the pH range of 6.5 to
20   8.7. Preferred inhibitors for particular enzymes are
included in Table 1.

The assay compound must be soluble in the aqueous
media. Solubility is measured by light scatter using
the percent transmittance of light (or absorbance)
25   through the mixture of the media and assay compound. As
measured on a spectrophotometer, the assay compound
should have a background color at a concentration to be
used in an assay of less than 1000, preferably less than
800, and most preferably less than 500 milliabsorbance
30   units at 340 nanometers (25 °C) blanked against
distilled or deionized water. The assay compound will
usually be used at a concentration of 0.5 to 10 mM. A
useful concentration for determining solubility is 5 mM.

Preferably, a two fold excess quantity of the assay
35   compound that will react with the enzyme during the time
of the assay must be soluble in the aqueous media. An
excess of assay compound is preferred. If an

insufficient amount of the assay compound is provided, the enzyme reaction will completely hydrolyze the assay compound and the dynamic range of the assay will be limited. The resulting indicator compound will have a
5   limited fluorescence duration. However, when an excess of the assay compound is employed, the enzyme reaction will continuously hydrolyze the assay compound and the fluorescence duration will continue during the enzyme reaction. This provides the advantage of having a
10 longer time period in which to sense for one or more reaction states of the assay compound.

A solubilizing agent can be utilized with assay compounds for which salts are not available, or where such solubilizing agents will assist the transfer of the
15 assay compound into a metabolically active cell. T h e solubilizing component is present in an amount effective to enable the assay compound to pass through the cell lipid bilayer without detrimentally affecting the cell. The solubilizing agent should be carefully chosen
20 because the wrong solubilizing agent can cause lysis or cell death.

When the assay compound has a background color (at the concentration to be used in an assay) greater than 1,000, greater than 800 or greater than 500
25 milliabsorbance units, a solubility component may be used to lower the background color to less than 1,000, less than 800 or less than 500 milliabsorbance units. However, the concentration of the solubilizing component is limited. If a high concentration of the solubilizing
30 component is used, metabolically active cells will be lysed. If a low concentration of the solubilizing component is used, sufficient solubility of the assay compound will not be attained. The effective amount of solubilizing component may be empirically determined,
35 but is typically less than 10.0 % by weight of the assay compound.

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Suitable solubilizing components include non-ionic surfactants, polyethylene glycol, dimethyl sulfoxide (DMSO), and mannitol, as noted in Table 2. BRIJ 35 and TWEEN 20 are the tradenames for products from ICI Americas, Inc. PLURONIC 25 R8 is the tradename for a product from BASF Wyandotte. TRITON X100 is the tradename for a product from Rohm and Haas Company.

Table 2

CONCENTRATION	COMMERCIAL NAME	CHEMICAL STRUCTURE
0.1%	BRIJ 35 (non-ionic)	Polyoxyethylene lauryl ether
0.2%	PLURONIC 25 R8 (non-ionic)	Ethylene oxide with hydrophobic base from propylene oxide and propylene glycol
0.1%	TRITON X100 (non-ionic)	Octylphenoxy polyethoxy ethanol
0.1%	TWEEN 20 (non-ionic)	Polyoxyethylene sorbitan monolaurate (polysorbate 20)
0.1% 5% 4.5%		Polyethylene glycol Dimethyl sulfoxide Mannitol

When using a solubilizing component, certain difficulties have been encountered. While the solubilizing component facilitates the transmission of the assay compound into the metabolically active cell, the solubilizing component will also facilitate the expulsion of the fluorescent indicator group compound from the metabolically active cell. The expulsion of

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the indicator group will have the negative effect of permitting non-enzyme containing cells to absorb free dye. When this occurs, the accuracy of an enzyme assay is compromised.

In addition, the electronic configuration and polar nature of the liberated indictor dye influences its ability to be retained within the cell. Retention of the dye is important for proper detection.

A feature of the present invention used to avert the problem of cellular expulsion when using a solubilizing component, is for the assay reagent to include a retention component. The retention component will comprise at least one agent that will inhibit a cell pump mechanism for expressing extracellular material. Such cell pumps include the multiple drug response pump, calcium channel pump, sodium pump, potassium pump and ATPase pump. Suitable retention components include trifluoperazine•HCl, prochlorperazine•maleate, and chlorpromazine•HCl to inhibit the multiple drug response pump; verapamil•hydrochloride to inhibit the calcium channel pump; and digoxin ($C_{41}H_{64}O_{14}$), digoxin derivatives, such as ouabain ($C_{29}H_{44}O_{12}$), and strophatidin ($C_{23}H_{32}O_6$) to inhibit the sodium, potassium and ATPase pump.

The media in which the assay compound is dissolved must be compatible with the cell so that the cell can remain metabolically active in the media for at least the duration of the assay. The media is preferably sterile and free of endotoxin and chemicals that adversely affect the physiology of the cell. The assay compound is preferably completely soluble in the media at the concentration at which it is used. The assay compound is preferably used in concentrations up to the saturation or the suspension level or before turbidity occurs. The media may be physiological saline or a buffered solution (phosphate buffered saline) in which the assay compound and other additives are dissolved.

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The media should preferably include a buffer agent so that the pH of the assay mixture of metabolically active cells and assay compound is maintained at a point that is appropriate for the enzyme hydrolysis.

5   For storage purposes the compound and media mixture should be lyophilized under conditions where sublimation of the solvent occurs upon application of a vacuum. Applying a vacuum to the sample at a temperature where a liquid forms on the solid before going to a gas phase,
10   referred to as "melt back" may cause degradation of the compound. Appropriate temperatures should be determined for each compound, and preferred temperatures are usually -5°C to -35°C for predominantly aqueous solutions. During the thermal cycle of lyophilization,
15   heat may be applied after sublimation to drive off any additional moisture. The product temperature should never exceed the heat applied and the product should be brought to room temperature over 15 to 72 hours. The vacuum should be returned to atmospheric conditions by
20   bleeding in dry nitrogen. The product is stoppered at atmospheric pressure and temperature. The lyophilized compound is stored at 4°C to 8°C and may be reconstituted using endotoxin-free deionized water.

       Auto-hydrolysis, which is the nonspecific
25   hydrolysis of the substrate, yields cellular fluorescence not derived from the target enzyme. Stability of the substrate compound has been demonstrated to be a key factor in preventing auto-hydrolysis.

30   The assay compound and/or the assay reagent should be sufficiently stable so that no auto-fluorescence or chemiluminescence is created by the degradation of the assay compound prior to cleavage by the enzyme. Preferably, when the assay compound or assay reagent is
35   stored at 20°C for 30 days, preferably 90 days, more preferably 180 days and most preferably one year, the reagent exhibits a photon count of 100,000 or less.

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Photons can be measured by using a 2 millimolar solution
of assay compound in deionized water and a path length
of 1 cm against a rhodamine 110 standard as previously
described. Fluorescent impurities should account for
5   less than 10% of the fluorescence generated during the
assay.

An acceptable reagent should have the following
three characteristics; (1) there should be a low level
of native free fluorescence that is absorbed by the
10   cells, non-specifically. Thus, there should be a low
level of fluorescent impurities such as free indicator
compounds. The acceptable and preferred levels of these
impurities have already been described. (2) The reagent
should be stable over time so that it does not need to
15   be used shortly after it is prepared.   Certain
impurities and certain reagent additives can increase
the rate of autohydrolysis which increases the
fluorescence of the reagent. Acceptable and preferred
stabilities have already been discussed.   (3) The
20   reagent should also have a high enough rate of reaction
with the enzyme being measured so that fluorescence
generated as a result of reaction between the enzyme and
the reagent can be easily measured. In one aspect, the
reaction rate should be sufficiently high that
25   fluorescence generated as a result of cleavage of the
leaving group inside the cell is at least 2 times,
preferably at least 10 times, more preferably at least
50 times and most preferably at least 100 times greater
than other non-specific fluorescence generated in the
30   assay. In another aspect, the reagent should contain an
unblocked assay compound which has a reaction rate which
is at least 2 times, preferably at least 5 times, more
preferably at least 100 times, most preferably at least
1000 times the reaction rate of a corresponding blocked
35   assay compound. For example, the unblocked assay
comp unds of the present invention which contain
unblocked amino and or peptide leaving groups have an

enzymatic reaction rate which is considerably greater
than the reaction rate of the corresponding compound
wherein the amine group(s) on the leaving group is
blocked by, for example, a Cbz group.

Types of Assays

It has been discovered that the assay reagent can
be used to determine enzymatic activity of metabolically
active whole cells to provide indication of the presence
of a disease, of the progress of a disease, the efficacy
of a drug, and cell differentiation.

It has been found that the activity of one or more
enzymes changes with disease progression. Changes in
the activity of one or more enzymes can be examined to
provide an indication of the presence and progress of a
disease. In addition, the measurement of the activity
of certain enzymes can provide an indication of the
response to certain drugs or treatments, since the
activity of one or more enzymes will change if the drug
is successfully fighting, modulating or treating the
disease. Still further, it has been determined that
differentiation of a cell can be determined by the
presence of one or more selected enzymes.

Existing tests for the presence of a disease,
progress of a disease, or efficacy of a drug require
significant extracellular concentrations of the enzymes
that are being measured. Usually, hours or days are
required to allow extracellular concentrations to rise
to detectable levels. The present invention has the
further advantage of providing a method to produce a
reagent for measuring the intracellular concentrations
of enzymes. This enables the diagnostic assay to obtain
analysis of the enzyme of interest in a shorter period
of time and to monitor intracellular events as they are
occurring.

Enzymatic assays are performed by contacting
metabolically active cells with an assay reagent. The

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leaving group is selected to be one which can be cleaved from the indicator group by the targeted enzyme. The reaction occurs for a period of time sufficient for the leaving group to be cleaved from the indicator group by

5   the targeted enzyme. Sensing for one or more reaction states confirms cleavage of the indicator group by the enzyme.

In some instances, one can characterize a disease, the progress of a disease, or efficacy of a treatment by

10 sensing for only a single enzyme, as in the case where a disease is characterized by the presence or lack of activity of an enzyme. For instance, Hereditary Non-Spherocytic Hemolytic Anemia (HNSHA) is distinguished from hereditary spherocytosis by the fact that red blood

15 cells are morphologically normal and manifest a normal osmotic fragility. Only in the case of pyrimidine 5'nucleotidase deficiency is the erythrocyte morphology changed to a basophilic stippling. (See Example 27) However, in most instances these conditions will be

20 characterized by assaying for the activity of at least two enzymes. In practice, five or more enzymes will be used in a panel to serve as checks and to reduce the probability of false positive or false negatives since the activity of a targeted enzyme can be present in two

25 different diseases. However, as the number of targeted enzymes are increased in the panel, the assay becomes more reliable or differentiable. The detection is more reliable because two different diseases will have different enzyme patterns.

30   Thus, there are at least two ways to run the assay of the present invention:

running a single assay and detecting a difference between the beginning state and the end state of a substrate, such as the cleavage of a single substrate by

35 a target enzyme to yield free peptide and fluorescent indicator dye; and

running a series of assays with a pattern matrix of several substrates reacted with an abnormal cell versus the same matrix reacted with a normal cell.

"Reliability" refers to the ability to make pattern matrix decisions without failure. Error in a single test may not, in fact, invalidate a pattern matrix. For a small set of assays, the assay provides an increased capability to differentiate states of abnormality.

The panel of selected enzymes are created by developing a range of normal values for enzymatic activities and ratios of enzymatic activities to each other. This panel will be used to compare the test results from the cell analyte. The enzyme activities from the examined cell analyte is compared to at least one of a reference/non-diseased cell or a reference/diseased cell to obtain an indication of a diseased state.

In other instances, the analysis of cellular enzymes involving classes of enzymes provides the ability to sort cells by type or morphology. As many as a thousand different enzymes may be operative in any given cell, but only a few dozen enzymes define the unique function or functions of any one cell type. Many enzymes are inhibited or missing from functionally different cells. Determination of proteases, glycosidases, glucosidases, carbohydrases, phosphodiesterases, sulfatases, thioesterases, pyrophosphatases, nucleotidases, nucleosidases, saccharidases, esterases, phosphatases, lipases and combinations thereof provides a matrix to rank cells by their functional activity.

Classification of normal cells morphologically can be made by determining key enzyme activities. For example, nucleated red blood cells (NRBC's) can be distinguished from non-nucleated red blood cells (RBC's) by determining dipeptidyl peptidase IV activity in the cell analyte. In NRBC's dipeptidyl peptidase IV activity will be present but in RBC's dipeptidyl peptidase IV activity will be absent. In addition, the age of RBC's can be determined by the presence or absence of adenosine deaminase or 5'nucleotidase.

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In still other instances, the analysis of cellular enzymes involving classes of enzymes provides the ability to study cell proliferation. Cell proliferation is stimulated by growth factors. Cell proliferation is

5   the ability of cells to divide and increase their numbers. Phases of cell division are under gene control and take a specified time period for each part of the division process. The time from one division to the next includes a randomly variable component. Different

10   cell types require different growth factors in order to divide. All cells compete for growth factors. Cells are programmed for a certain number of divisions and then they die.

Abnormal cells that disobey normal constraints on

15   cell division proliferate to form tumors in the body. They also appear transformed in cell culture. Cell transformation is often accompanied by mutation or over-expression of specific oncogenes.

Some normal cells proliferate as part of their

20   function. Signaling molecules are produced in the course of the inflammatory response and stimulate the bone marrow to produce more leukocytes. This regulation tends to be cell type specific. More specifically, some bacterial infections cause a selective increase in

25   neutrophils, while infections from parasites cause a selective increase in eosinophils.

A blood cell differential can be constructed using this invention to determine cell types, immature cells, mature cells, abnormal cells due to drug interreaction

30   and abnormal cells due to disease. For example, cell types can be identified by the assay of the present invention. For example, lymphocytes can be distinguished from monocytes or neutrophils by peroxidase activity. Lymphocytes will not show

35   peroxidase activity while neutrophils will show peroxidase activity, and lymphocytes will not show esterase activity while neutrophils will show esterase

activity. In addition, acetate esterase activity is present in monocytes that have been inhibited with sodium fluoride, but absent in neutrophils.

5 In addition, the analysis of cellular enzymes involving classes of enzymes provides the ability to study cell activation. Activation of T cells is a complex process involving various secreted proteins called interleukins which act as chemical mediators. Activation is thought to begin when the T cell 10 stimulates the antigen presenting cell to secrete one or more interleukins. IL-1 mediator causes the T cell to stimulate its own proliferation by inducing it to secrete a growth factor IL-2, as well as synthesize IL-2 receptor to initiate proliferation.

15 Helper T cells are essential for B cell antibody response. Once activated by foreign antigen, the T cell presents the antigen to a B cell for antibody synthesis. Other Helper T cells secrete γ interferon which attracts macrophages and activates them to defend against 20 infection by microorganisms. Diagnosis of infection from inflammation and inflammatory diseases has been achieved using the assay reagent in a pyrogen-free, sterile environment. Activation and proliferation agents, like phorbol myristate acetate (PMA), f-Met-Leu-25 Phe, IL-1, IL-2, GMCSF and γ interferon are added to the media and specific cell types are observed for response. Treatment regimes can also be monitored for effectiveness by using the assay reagent in conjunction with growth stimulators or signal peptides.

30 The present invention has potential use in the following clinical applications: diagnosis of cervical cancer, diagnosis of viral replication in HIV patients, diagnosis of HIV infected blood in blood supply, diagnosis of TB infected HIV patients, improved blood 35 differential, differential diagnosis of viral from bacterial infections, differential diagnosis of Lupus from rheumatoid arthritis, differential diagnosis

between rheumatoid arthritis from osteo arthritis, diagnosis of vasculitis, diagnosis of cardiovascular disease, monitoring of chemotherapeutic efficacy, diagnosis of Hodgkins Disease, confirmation of gene

5 implantation and diagnosis of transplant rejection.

For a diagnosis of cervical cancer, several enzymes related to the presence of cervical cancer can be measured.

For a diagnosis of viral replication in HIV

10 patients, HIV replication in blood cells can be monitored. A sensitive measure of HIV replication can be important as a predictor of rapid movement into the AIDS state from the HIV infected stage of the disease. Since the virus replicates in the lymphocytes and

15 monocytes, monitoring specific enzyme levels can make the monitoring both inexpensive and reproducible.

Identification of infected units in blood supply is one of the major goals of those responsible for the quality of blood supplied for transfusion to reduce the

20 probability of HIV or Hepatitis infection. A low cost screening methodology can be devised whereby the blood can be subjected to HIV antibody testing and testing by the method of this invention.

In the management of AIDS patients, the early

25 diagnosis of Tuberculosis is important to insure rapid recovery and to reduce the chance of further complications. The objective of such a test using this invention is to distinguish TB$^+$HIV patients from TB$^-$HIV patients. The early identification of the TB$^+$HIV

30 patients can permit administration of therapy to prevent additional complications in these immune deficient patients.

This invention also has utility for the differential diagnosis of viral from bacterial

35 infections. Many patients have an elevated temperature and it is not known whether the temperature is from a viral or bacterial origin. The differential diagnosis

between viral and bacterial infections assists the clinician in the management of these patients by allowing the physician to apply the proper therapy on an as needed basis.

5   This invention has further utility for differential diagnosis of Lupus from rheumatoid arthritis/drug monitoring in rheumatoid arthritis and Lupus patients. In the early course of disease, the symptoms for Lupus Erythematosis and rheumatoid arthritis are sufficiently

10   similar that differential diagnosis of the disease is difficult, especially when a Lupus patient has early arthritic involvement. This has clinical consequences since it delays the administration of the correct therapy. Lupus can be a clinically aggressive disease

15   and it is beneficial to the patient to have the correct diagnosis at an early date. These patients have different enzymes in activated states meaning that this methodology is the modality to use for a differential diagnosis. Additionally, monitoring the therapeutic

20   application of steroid drugs can be of benefit to the patient.

        This invention has still further utility for differential diagnosis between rheumatoid arthritis from osteo arthritis. Rheumatoid arthritis is an aggressive

25   autoimmune disease which results in destruction of the panus of the joint. Osteo arthritis is a degenerative disease of the aging joint which is not immune mediated. Since immune cells migrate throughout the body, this methodology provides an early differential diagnosis

30   between these two diseases. This is important since the correct therapy for each disease is different.

        Moreover, this invention has utility for diagnosis of vasculitis. Vasculitis is an autoimmune disease of blood vessels generally in the extremities. Patients

35   with this disease typically have nondescript complaints of pain which do not permit diagnosis until considerable damage has been completed on the vascular system by the

immune cells. Since it is an autoimmune disease caused
by circulating immune cells, the disclosed methodology
can provide the needed information to make an early
diagnosis.

5   Furthermore, this invention has utility for
monitoring of cardiovascular disease. Atherosclerosis
results in the deposition of platelets and other
cellular components into the walls of coronary vessels.
This process results in the loss of elasticity of the
10   vessels and eventually in death. It has been shown that
in these patients, as many as 20% of the platelets are
in the activated state. Evaluation of platelets can
permit the identification of patients with active
atherosclerotic   processes   ongoing   and   permit
15   administration of disease altering drugs.

Moreover, this invention has utility for monitoring
of chemotherapeutic efficacy. Patients undergoing
chemotherapeutic therapy have altered enzyme patterns
which indicates that this change in enzyme levels can be
20   used to monitor the effectiveness of chemotherapy.

In addition, this invention has utility for
diagnosis of Hodgkins disease. The practice of this
invention can be useful to monitor the stages of
Hodgkins disease.

25   Furthermore, this invention has utility for
diagnosis of transplant rejection. The practice of this
invention can be useful to monitor the acceptance of an
organ   transplant.   All   patients   are   given
immunosuppressants to prevent organ rejection and
30   therefore it is difficult to distinguish infection from
rejection.

Moreover, this invention has utility for monitoring
for metastatic invasion. It has been found that tumor
cells have different patterns of enzymes from normal
35   cells in the same tissue. Identification of the types
of enzymes is useful and important for predicting
metastatic potential and invasion. Tumor cells in
circulating blood can be useful to predict the
progression of the disease.

Preparation of Metabolically Activ Whole Cells

The assay reagent is reacted with a metabolically active whole cell analyte. The metabolically active whole cells are contained in tissue, blood, cell cultures or other cells containing constituents. Preferably, the metabolically active whole cells are separated into cell types. The metabolically active cells to be analyzed are isolated by known techniques such as differential lysis, differential centrifugation, and affinity columns. However, separation of the cells to be studied from other cells is not always essential.

The cells are usually washed to remove any extracellular enzymes, optionally with lysis or physical separation of unwanted cells. Several preferred techniques for accomplishing this are summarized in Figs. 1A-1D.

The analysis of the segregated metabolically active cells provides specificity for a particular enzyme analysis. For example, when the metabolically active cell is a leukocyte blood cell, the method comprises separating the leukocyte cell from the cell analyte, washing the remaining leukocyte cell to remove any serum or plasma enzymes, contacting an assay reagent compound with the leukocyte cell, and determining fluorescence from the leukocyte cell (See Fig. 1B). A modification of this method comprises washing the cell analyte to remove any serum or plasma enzymes, contacting an assay compound with the cell analyte, separating the leukocyte blood cells from the cell analyte, and determining fluorescence from the leukocyte cells (See Fig. 1A). In addition, another method that can be used for cell analytes of leukocyte blood cells, nucleated erythrocyte blood cells and platelets analytes comprises washing the cell analyte to remove any serum or plasma enzymes, contacting an assay compound with the analyte and determining fluorescence from the analyte (See Fig. 1C).

To confirm that cells are metabolically active at the time of the assay, it is desirable that the viability of the cells be checked at the time of the assay. Several tests are useful to determine the viability of cells. Trypan blue is a blue stain which diffuses into the cell and is removed by cells if the cells are viable. Dead cells will not remove the dye and will take on a blue color. Propidium iodide is a DNA-RNA stain which, if the cell is dead and membranes are damaged, will penetrate the cell and stain the DNA-RNA. Fluorescein diacetate-propidium iodide will cause living cells to take on a green color because the fluorescein diacetate will be hydrolyzed, while dead cells become red from the propidium iodide. Red blood cells do not undergo cell division, and therefore a test for the presence of 2,3-diphosphoglucose dehydrogenase (which is an indicator of cell division) is a useful test for viability.

The assay of the present invention is particularly useful for measuring intracellular concentrations of enzymes in mammalian cells such as human cells. However, the assay should also be useful in various or other types of cells which have metabolic activity.

Assay Conditions

The concentration of the cells in the media should be high enough to provide a reading of the desired number of cells within the desired time period, taking into consideration the speed of the instrument that is being used. For current flow cytometry techniques, a concentration of about three million cells per milliliter is appropriate to yield a measurement of about 10,000-15,000 cells in about 1-2 minutes.

The assay compound is generally employed in concentrations in excess of the amount which can be completely hydrolyzed by the quantity of enzyme within the time of the assay. An assay compound concentration

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that is too high may have a negative affect on enzyme
activity, since the leaving group can be a negative
feedback inhibitor to enzyme activity.

5   The leaving group concentration in a cellular
optimization is determined using Km (a known rate
constant) and V_{MAX} (maximum velocity) calculations. The
leaving group is preferably present in an amount from
about 2 to about 100 x V_{MAX} and most preferably from
about 2 to about 10 times the amount which can be
10   completely hydrolyzed by the enzyme within the duration
of the assay period. Preferred leaving group
concentrations for particular enzymes are included in
Table 1.

The assay may be conducted either as a rate
15   determination or as an end point determination. Rate
determinations are preferred, because they are generally
less affected by auto-fluorescence. Consequently, a
rate determination assay is more sensitive and precise.
In a rate determination, the fluorescence of the assay
20   compound-cell analyte mixture may be determined promptly
after the cell analyte is contacted with the assay
compound. The ability to see a signal and distinguish
it from background noise determines the initial starting
point of data collection and the final data point is
25   preferably determined at the point where the slope of
the reaction rate changes, typically more than 2%.

Most cellular reactions do not strictly obey zero-
order kinetics. Most cellular enzymes show a delay
between the time of exposure of the cells to the assay
30   compound, and the ability to detect a signal that is
greater than the background noise. Cellular enzymatic
reactions that do not obey zero order kinetics are still
useful measurements as first order, pseudo first order,
or initial rate measurements. Multiple enzymes in a
35   reaction (mixed reactions) are displayed by slope
changes during the time course being monitored.

In an endpoint determination, the enzyme hydrolysis reaction is allowed to proceed for a predetermined length of time, usually at V_{MAX}. The reaction time can be calculated based on whether the reaction is zero
5   order or first order kinetics using Michaelis - Menton methodology. Alternatively, the reaction time can also be adjusted by a different elapsed time for pseudo-first order reactions.

It has been determined that a number of factors
10   will decrease the reliability of the assay, and yield false positive, or erroneous indications of enzymatic activity. These include (i) extended reaction between the cell analyte and the assay compound; (ii) another, non-targeted enzyme that is cleaving the leaving group;
15   (iii) auto-hydrolysis of the assay compound; (iv) inhibitors or stimulators that are present and undetected; (v) cells that are no longer metabolically active, or dead; (vi) mixed populations of cells; (vii) a transfusion of the patient before sampling; (viii)
20   non-specific dye uptake by negative cells; and (ix) background fluorescence. The creation of false negatives, or false indications of a lack of enzymatic activity, can be caused by (i) insufficient reaction between the cell analyte and the assay compound, (ii) a
25   hypoosmotic media leading to a decrease in cell activity; (iii) a cell that is no longer metabolically active; (iv) burst cells; and (v) the presence of inhibitors to the target enzyme.

It has been further determined that assays will be
30   significantly improved if reaction conditions are adjusted to maximize the activity of the assayed enzyme relative to other non-assayed enzymes which might otherwise compete for the leaving group. More specifically, the targeted enzyme can be involved in a
35   chain cascade reaction of enzymes sequentially coupled to other enzymes, as in a multi-enzyme reaction cascade.

The reaction conditions can be adjusted to maximize the efficiency of the pathway, or to decrease the efficiency of competing pathways. Such conditions preferably include at least one of pH, choice of form of
5 assay compound, temperature, osmotic pressure, ionic strength, and reaction time.

The pH at which an enzyme is most efficient can be determined from the literature, or determined empirically. As shown by Figure 2, pH maxima can have
10 two peaks (optima). Therefore, the selection of the appropriate pH should be made with care. In addition, care must be exercised when using pH information from the literature, because these values will usually be based on cytosol studies and not on intact,
15 metabolically active whole cells. Therefore, it is preferable to use values from the literature only as a starting point, and then to determine the appropriate pH from this reference point. Generally, the pH will be between about 4.0 and 9.5. The pH of the assay mixture
20 is controlled by dissolving the cell analyte and assay compound in an appropriate buffer. A list of preferred pH's for particular enzymes is included in Table 1.

The form of assay compound can be important since some enzymes require non-derivatized, natural structures
25 for recognition of binding and reaction, whereas other enzymes are less selective. More specifically, derivatization and salt formation of the assay compound are important properties for solubilization, enzyme recognition and protection from auto-hydrolysis.

30 A reaction run using the same data collection window without the enzyme source will determine auto-hydrolysis of the substrate and therefore the potential for negative cells to absorb the dye non-specifically resulting in false positive.

35 The time of the assay is typically less than 30 minutes, preferably less than 20 minutes, usually between 5 seconds and 20 minutes, and most preferably

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between about 10 seconds and about 5 minutes. Some
enzyme systems, such as esterase and phosphatase, can
react with the assay compound in shorter periods of time
due to concentrations of enzymes found in the cell. The
5   reaction time should be limited so that the effects of
cellular expulsion of the indicator compound will be
avoided. Preferred time periods for assaying particular
enzymes are included in Table 1.

The temperature at which the assay is performed
10   must be physiologically acceptable to the cell. The
temperature must be high enough to retain viability and
to ensure enzyme activity, but not so high as to cause
degradation or other deleterious reactions involving the
leaving group, the enzyme, or other components of the
15   mixture. Particular enzymes, or enzymes in particular
pathways, are more reactive at particular temperatures.
The temperature is preferably maintained between about
30°C to about 40°C, more preferably between about 35°C
and about 38°C, and most preferably between about 36°C
20   to about 38°C. Preferred temperatures for a variety of
enzymes are shown in Table 1.

The osmotic pressure of the assay mixture is
controlled to be within physiological ranges from about
250 milliosmoles to 350 milliosmoles, preferably from
25   about 275 milliosmoles to 320 milliosmoles. The osmotic
pressure must be selected to maintain the viability of
the metabolically active whole cell. Variations in
osmotic pressures will result in lysis of the cell,
severe shrinking or shriveling (crenation) when too low,
30   and swelling or bursting (stomatolysing) of the cell
when too high.

The ionic strength of the assay mixture should be
selected so as to avoid shriveling crenating or bursting
(stromatolysing) of the cells, and also to maximize the
35   activity of the assayed enzyme relative to other, non-
assayed enzymes. An ionic strength that is too low
could deplete metals such as Ca^{+2}, Mg^{+2}, and Zn^{+2}, or

cause insufficient amounts of anions such as Cl^{-1}, NO_3^{-1}, SO_4^{-2} and PO_4^{-3} which are the cofactors that can be used to improve enzymatic activity. The ionic strength of the assay reagent is preferably between about 0.1 to 0.3 μ.

5 A list of preferred ionic strength values for particular enzymes is included in Table 1.

The fluorescence reading is made after the reaction has occurred or after a specific period of time. Typically, the reaction is stopped by immersing the

10 reaction container in ice and water which cools the cells to about 0°C. Sensing for one or more reaction states by fluorescence determinations confirms cleavage of the indicator group by the enzyme.

The fluorescence determinations are performed on a

15 Image Analysis System (IAS) or a Flow Cytometer (FC). The IAS is a microscope based system that measures fluorescence known to those skilled in the art. A representative example of an IAS is the Metamorph ™ by Universal Imaging Corporation, West Chester,

20 Pennsylvania. The structure and operation of flow cytometers is also well documented in the literature. Alternatives to traditional FC include slit-scan FC and stopped-flow FC. The type of instrument used to conduct the experiments described in the examples was a flow

25 cytometer (for example, a Coulter Profile® flow cytometer manufactured by Coulter Corporation of Miami, Florida). This flow cytometer measures fluorescence across the entire cell. Flow cytometric methods which measure fluorescence in only a part of the cell, such as

30 slit scan flow cytometry, have significant utility in the invention because the background fluorescence is significantly reduced when measurements are focused on the region of the cell where the enzyme is located.

The fluorescence determinations can also be taken

35 by a spectrofluorometer which has the capability to measure the very low fluorescence levels that are generated by the assay. The spectrofluorometer is tuned

to the excitation and emission wavelengths of the particular indicator being used. Preferred compounds such as rhodamine 110 and fluorescein have excitation and emission wavelengths of about 495 to 498 nm

5   (excitation) and 520 to 525 nm, respectively. The Model 8000C photon counting spectrofluorometer manufactured by the SLM company, a subsidiary of Milton Roy (Chicago, Illinois) was used.

        The flow cytometer can perform additional
10   measurements in addition to a single wavelength fluorescence measurement. The flow cytometers can be equipped to measure fluorescence at two or more separate wavelengths. Such readings are useful to perform assays according to the invention when using more than one
15   assay compound, or for using cell surface markers, such as monoclonal antibodies, to determine cell morphology. Additional wavelengths are useful to measure the activity of another enzyme, which can be a peptidase or a different enzyme such as a phosphatase, saccharidase,
20   nucleotidase, esterase, or lipidase. Such additional tests are useful for simultaneously characterizing disease states, and for determining cell morphology and cell types.

Assay Protocols

25   Preferred sample preparations by which enzymes can be assayed using the reagents prepared according to the method of the invention have been developed. These sample preparations can be modified, and are included herein to disclose those procedures that are currently
30   preferred.

        The practice of the cell probe assay is divided into three parts: 1. Sample preparation, 2. Data Collection (i.e., Detection of Fluorescence) and 3. Results (i.e., Data Analysis).

1. Sample Preparation:

Sample preparation can be divided into four different processes A, B, C and D which are illustrated in Figs. 1A, 1B, 1C and 1D, respectively. The choice of
5   sample preparation is dependent upon the user and the analyte. The four processes are:

        Process A: Examination of leukocytes or tissue cells with erythrocyte contamination with post-lysing.

10   A sample, consisting of whole blood (in EDTA, Heparin or ACD) or dissociated tissue or body fluids (synovial fluid) or cell culture media is obtained and stored in a manner so as not to decrease viability. The sample is washed sufficiently to remove plasma, media,
15   body fluid, debris and extra-cellular enzymes. The wash media consists of a physiologically balanced buffered salt solution. The washed cells are incubated at 37°C. 50 μL of sample and 25μL of substrate media are mixed together and allowed to incubate at 37°C for a
20   predetermined amount of time. At the end of the incubation period, unwanted cells are lysed with a lytic reagent, i.e., erythrocytes are removed. Compatible lytic systems are Q-Prep ™, an acid lyse (formic acid/quench), Erythrolyse ™, (acid lyse/detergent/
25   quench) or hypotonic ammonium chloride. The sample is then measured for fluorescence. The referenced lytic systems are commercially available from Coulter Corporation, Miami, Florida.

        Process B: Examination of leukocytes or tissue
30   **cells with erythrocyte contamination with pre-lysing.**

    A sample, consisting of whole blood (in EDTA, Heparin or ACD) or dissociated tissue or body fluids (synovial fluid) or cell culture media is obtained and
35   stored in a manner so as not to decrease viability.

Unwanted cells, i.e. erythrocytes, are lysed with a lytic reagent. Compatible lytic systems are acid lysed (formic acid/quench), IVCS lyse (quaternary ammonium salts)/quench or hypotonic ammonium chloride. The sample
5   is washed sufficiently to remove plasma, media, body fluid, debris and extra-cellular enzymes. The wash media consists of a physiologically balanced buffered salt solution. The washed cells are incubated at 37°C. 50 μL of sample and 25μL of substrate media are mixed
10 together and allowed to incubate at 37°C for a predetermined amount of time. At the end of the incubation period, the sample is then measured for fluorescence.

         Process C: Examination of platelets, erythrocytes,
15   **leukocytes, dissociated tissue, body fluids and**
         cell culture media.
         A sample, consisting of whole blood (in EDTA, Heparin or ACD) or dissociated tissue or body fluids (synovial fluid) or cell culture media is obtained and
20 stored in a manner so as not to decrease viability. The sample is washed sufficiently to remove plasma, media, body fluid, debris and extra-cellular enzymes. The wash media consists of a physiologically balanced buffered salt solution. The washed cells are incubated at 37°C.
25 50 μL of sample and 25μL of substrate media are mixed together and allowed to incubate at 37°C for a predetermined amount of time. At the end of the incubation period, the sample is then measured for fluorescence.

Process D: Examination of platelets, erythrocytes,
leukocytes, dissociated tissue, body fluids and
cell culture media using a mechanical separation to
isolate a cell population.

5   A sample, consisting of whole blood (in EDTA,
Heparin or ACD) or dissociated tissue or body fluids
(synovial fluid) or cell culture media is obtained and
stored in a manner so as not to decrease viability. A
mechanical separation to isolate a specific cell
10   population is performed, i.e., ficoll, differential
centrifugation, differential precipitation. The sample
is washed sufficiently to remove plasma, media, body
fluid, debris and extra-cellular enzymes. The wash
media consists of a physiologically balanced buffered
15   salt solution. The washed cells are incubated at 37°C.
50 μL of sample and 25μL of substrate media are mixed
together and allowed to incubate at 37°C for a
predetermined amount of time. At the end of the
incubation period, the sample is then measured for
20   fluorescence.

2. Detection of Fluorescence:

          The instruments used to detect fluorescence are the
flow cytometer or fluorescent microscope. There are
four different instrument configurations for the flow
25   cytometer, A, B, C and D.   Any of the four
configurations can be used with any one of the sample
preparations described above. The choice of which
configuration is selected is dependent upon the user and
the information sought to be obtained. The four
30   configurations are:

Configuration A:

          Configuration A analyzes the cells by size,
granularity and single color.   In the first
configuration, the flow cytometer separates the cells by
35   size and granularity. The activity of an enzyme is then

assayed using the reagent compound. Two samples are
allowed to proceed at different times and the reaction
is stopped. The difference in fluorescence permits the
calculation of a rate. Total population counts
5   preferred are 20,000 to 500,000 cells. Use of light
scatter or hematology parameters provide size and
granularity separation. Intensity bitmap of desired
populations and determination of fluorescent activity by
single measurement point or multi-point measurement can
10   be employed. Determine count, percentage and
fluorescent intensity of a multi-modal population
representing enzymatic activity.

Configuration B:

Configuration B analyzes the cells by size,
15   granularity and two colors. In the second
configuration, the flow cytometer separates the cells by
size and granularity. Cell morphology is determined by
a fluorescence assay with a monoclonal antibody marker.
The rate of the hydrolysis of the assay compound is then
20   determined. Total population counts preferred are
20,000 to 500,000 cells. Use of light scatter or
hematology parameters provide size and granularity
separation. Intensity bitmap of desired populations and
determination of fluorescent activity by single
25   measurement or multi-point measurement can be employed.
Determine count, percentage and fluorescent intensity of
a multi-modal population representing enzymatic
activity. The analysis is a 2-color analysis measuring
enzymatic activity in one color and surface-marker
30   antibody cell morphology in the other color.

Configuration C:

Configuration C analyzes the cells by size,
granularity, two colors and backgate fluorescence.
Configuration 3 is a modification of the Duque method.
35   Duque, R. E., "Flow Cytometric Analysis of Lymphomas and

Acute Leukemias", <u>Annals of the New York Academy of
Sciences, Clinical Flow Cytometry, 677</u>, pp. 309-325
(March 20, 1993). The size and granularity of the cell
are separated by a flow cytometer using light scatter
5   and/or with surface markers, such as monoclonal
antibodies. A series of cell populations are
determined, with rearrangement of the histogram to
identify the disease and normal cells. The activity of
the enzyme is then assayed. Total population counts
10   preferred are 20,000 to 500,000 cells. Use of light
scatter or hematology parameters provide size and
granularity separation. Intensity bitmap of desired
populations and determination of fluorescent activity by
single measurement point or multi-point measurement can
15   be employed. Determine count, percentage and
fluorescent intensity of a multi-modal population
representing enzymatic activity. The analysis is a 2-
color analysis measuring enzymatic activity in one color
and surface-marker antibody cell morphology in the other
20   color. Backgate fluorescence data on size and
granularity to determine count and percent of diseased
cells.

        Configuration D:
        Configuration D analyzes activity of a population
25   of cells over time. Total population counts preferred
are 20,000 to 500,000. Use of light scatter or
hematology parameters provide size and granularity
separation. Intensity bitmap of desired populations and
determination of fluorescent activity by single
30   measurement point or multi-point measurement can be
employed. Determine count, percentage and fluorescent
intensity of a multi-modal population representing
enzymatic activity. The analysis is a 2-color analysis
measuring enzymatic activity in one color and surface-
35   marker antibody cell morphology in the other color.

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3. Data Analysis:

The measured fluorescence intensity can be converted from mean channel fluorescence (in peak or integrated mode) to MESF (molecules of equivalent soluble fluorochrome, Flow Cytometry Standards Corp., San Juan, Puerto Rico) or International Units of hydrolysis per cell. A normal range of enzyme activity is established by assaying males and females in sufficient quantity to characterize the population levels statistically.

Various disease states are assayed for enzymatic activity and compared to the normal range. Three conditions will exist from this data:

1. Obvious increases or decreases in enzyme levels outside the normal range

2. Patterns of enzyme activities representing morphology

3. Patterns of enzyme activities representing disease states

Artificial intelligence or Non-Negative Least Squares (NNLS) programs and analysis of variance (ANOVA) programs are useful in identifying patterns of enzyme activities representing morphology, cell types and patterns of enzyme activities representing disease states.

A first, and most obvious technique for disease diagnosis is identifying the absence or presence of a single enzyme. One example of such a single enzyme diagnosis is the diagnosis for Gaucher's disease, which is diagnosed depending on the lack of a particular enzyme, namely, glucocerebrosidase.

The remaining techniques treat the absence or presence of a combination of enzymes as a complex interplay of metabolic systems, wherein each cell contains a group of enzymes, and the concentrations of these groups of enzymes are gaussian distributed, having a normal range or values, wherein values in disease states fall outside the normal range.

Analysis of Variance (ANOVA)

There are two steps in the mathematical analysis of the data. The first step is to analyze the variance in the data. The purpose of the analysis is to identify which combination of enzyme concentrations for various cell types are diagnostic of particular disease states or treatment modalities. (For simplicity, disease states and treatment modalities will be referred to collectively as disease states, henceforth).

To analyze the variance, each set of enzyme concentrations for each enzyme measured for each cell type is considered as a component of a composite measurement vector. A data matrix, such as the Full Covariance Data Matrix in Fig. 14A, including columns of basis measurement vectors known to characterize certain disease states is generated. The rows of the matrix represent the measurement vector components for each disease state under consideration. A variation across a row indicates that the various disease states affect cell metabolism such that the concentration of that enzyme in that cell type is changed; obviously such a difference provides information about the underlying disease state.

The data matrix needs to be developed for different disease states. Patients for the disease state data matrix can be first identified using conventional technologies. The disease state matrix can be expanded to include differentiation of stages of the disease as well as the influence of drug pharmokinetics on cellular function. Separate studies of drug pharmokinetics on human tissue culture cell lines can be performed to provide a reference.

The metabolism controlled by some enzymes in some cell types will be insensitive to disease state. Others will react collectively in a complex pattern with different enzymes in certain cells to produce a pattern of enzyme concentrations that will definitively

characterize a particular disease state. The analysis
of variance is necessary to select out that combination
of enzymes in particular cells which are most useful in
distinguishing among the various disease states spanned
5   by the basis measurement vectors.

The selection of a relatively small number of
components for the measurement vector is necessary to
simplify the later analysis, reduce the number of
physical measurements which must be made and to reduce
10   the effects of spurious noise generated from the
measurements and from individual variation among the
same population. An example of the selected number of
components is illustrated in Fig. 14A as the Reduced
Covariance Data Matrix of Strongly Contributing Factors.

15   **Squared Deviations From the Mean**
The simplest way to analyze the variance is to
compute the variance across the row for each row in the
data matrix. Those rows with a high variance correspond
to enzymes whose concentration in a particular cell type
20   varies most strongly across the disease states under
consideration. This method of analysis neglects any
interaction of various enzymes with each other; however,
it provides a simple, gross indication of which enzymes
in which cell types are most affected by the disease
25   state. This technique was used in the "Variance" column
of Table 5.

**Eigenvector or Principal Components Analysis of
Variance**
A more complete way to analyze variance is to
30   compute the eigenvalues and eigenvectors of the data
matrix, as described for example in J.D. Jobson, Applied
Multivariate Data Analysis, Springer Verlag N.Y. (1992).
Such an analysis is conventionally termed an eigenvector
analysis or a principal components analysis (PCA). In
35   practice, rather than on the data matrix, D, itself, the
eigenvect r analysis is performed on the covariance

matrix of the data $(D-m)^t(D-m)$, where the superscript t refers to the transpose of the matrix and m is the vector whose components are the mean of each row of D.

Each resulting eigenvector includes a particular weighted combination of the measurement vector components which act in concert with each other. Each eigenvector has a corresponding eigenvalue which is proportional to the total variance in the measurement which is accounted for by that eigenvector. Each eigenvector is also orthogonal or independent of every other eigenvector. This technique was used in the "Eigenvector 1" and "Eigenvector 2" columns of Table 5.

For the data matrices here, if the eigenvectors are arranged in order of decreasing eigenvalue, the vast majority of the variance is accounted for by the first several eigenvectors. Thus, the principal factors distinguishing the various disease states under consideration can be captured with a small number of combinations of the enzyme concentrations in the various cell types. Fig. 14A illustrates the process of developing the reduced covariance data matrix by eigenvector analysis.

An examination of the combination of measurement vector components contributing most to distinguishing different disease states, in light of the metabolic pathways linking the various enzymes, can be used to understand the underlying metabolic changes occurring with the various disease states. The foregoing eigenvector analysis is used to select from the entire series of measured enzyme concentrations for different cell types, the combination of enzymes which is most useful in characterizing the disease state.

Diagnosing Disease State, Based on Measurement

NNLS

Once the measurement vector and data matrix are
reformed with the selected (reduced number of) enzyme
5   concentrations for particular cell types, measurements
of patients with unknown diseases (presumed to be within
the basis disease states of the data matrix) can be used
to diagnose their disease state.   Two methods are
described here to accomplish the second step of the
10   mathematical analysis of the data, the inference of
disease state from the measurement vector: a Non-
Negative Least Squares based algorithm (NNLS) and a
neural net.

NNLS is a non-negatively constrained least squares
15   solution to the problem of determining the disease state
from the reduced measurement vector.   The algorithm,
which is disclosed in C.L. Lawson and R.J. Hanson,
Solving Least Squares Problems, Prentice-Hall N.J.
(1974) finds that linear combination of the basis
20   measurement vectors which most closely fits, in a least
squares sense, the measurement vector of the patient
whose state is being diagnosed.   The resulting solution
is a vector the magnitude of whose components reflect
the probability that the unknown disease state is each
25   one of the basis disease states.   The algorithm
constrains the components of the solution vector to be
non-negative; this constraint can be applied because the
components of the vector represent probabilities, which
by definition must be non-negative.   The non-negativity
30   constraint is extremely important in stabilizing the
solution to this often mathematically ill-conditioned
inversion problem.

Ideally, the solution vector has only a single non-
zero component, in this case, the disease state
35   corresponding to the chosen basis measurement vector is
the diagnosis of the patient.   Because of noise in the
measurements and individual biological variation among

individuals or because a patient is afflicted with more than one disease at a time, the solution vector provides a range of possibilities for the diagnosis; the component with the highest magnitude representing the

5   most probable disease state, etc. Fig. 14B illustrates the process of extracting predicted disease state probabilities from the reduced covariance data matrix.

       As illustrated in Fig. 14A, a database is obtained for both normal states and various disease states. An

10   example of an array of values obtained for 5 cell types (lymphocytes, monocytes, platelets, granulocytes and erythrocytes) using 50 different enzyme assays is shown in Table 3A-3C. This Table further gives a mean value, two standard deviation low value and two standard

15   deviation high value for each cell for each enzyme.

TABLE 3A

NORMAL LEUKOCYTE ENZYME ACTIVITY				
			LYMPHS	
ENZYME	SUBSTRATE	MEAN	LO	HI
Aminopeptidase	LEU	34.94	14.73	55.14
Aminopeptidase M	ALA	75.85	47.46	104.25
Pro Aminopeptidase	PRO	1.70	0.53	2.88
Aminopeptidase M	LYS	1.16	0.33	1.99
Aminopeptidase M, N	GLY	18.18	7.21	29.14
Aminopeptidase N	SER	1.35	0.28	2.41
Endopeptidase I	ARG	2.02	0.67	3.38
Endopeptidase I	ARG-TFA	1.47	0.50	2.45
Aminopeptidase A	ASP	0.26	0.04	0.49
Cathepsin B	VS	3.81	0.57	7.06
Cathepsin B	VS-M	3.78	0.86	6.70
Cathepsin B	VK	1.87	0.05	3.69
Cathepsin B	VK-M	2.05	0.00	4.40
Cathepsin B	QS	1.70	1.06	2.34
Cathepsin B	QS-M	1.14	0.67	1.61
Cathepsin B	LG	13.83	6.56	21.09
Cathepsin B	LG-M	4.71	1.33	8.08
Dipeptidylpeptidase II	KA	11.47	3.00	19.93
Dipeptidylpeptidase II	KA-M	0.55	0.30	0.80
Dipeptidylpeptidase IV	Z-AA	151.20	113.68	188.73
Dipeptidylpeptidase IV	Z-AA-M	94.47	47.80	141.14
Dipeptidylpeptidase IV	Z-GP	107.56	72.84	142.28
Dipeptidylpeptidase IV	Z-GP-M	53.74	29.05	78.44
Cathepsin D	GL	9.42	3.84	15.00
Cathepsin D	GL-M	2.56	0.00	6.34
Cathepsin C	Z-AG	15.17	7.48	22.86
Cathepsin C	Z-AG-M	14.24	2.97	25.52
Dipeptidylpeptidase IV	AA-TFA	56.95	3.01	110.89
Dipeptidylpeptidase IV	AA-M	9.88	0.00	24.56
Cathepsin C	Z-TP6.5	21.97	8.98	34.96
Cathepsin C	Z-TP6.5M	20.62	9.03	32.22
Cathepsin B	LLR	4.09	0.00	9.90
Cathepsin B	LLR-M	3.38	0.50	56.26
Cathepsin B	LGLG	1.42	0.76	2.08
Cathepsin B	LGLG-M	1.34	0.38	2.29
Esterase	FDA	13.38	0.00	27.21
Monocytic Esterase	FDA-NAF	17.98	0.43	35.54
Peroxidase	DCFH-DAMES	4.11	0.68	7.53
Activated Peroxidase	DCHFMESPMA	4.69	0.98	9.41
Collagenase	GPLGP	8.97	3.64	14.30
Collagenase	GPLGP-M	7.89	2.40	13.37
Collagenase	GFGA	0.36	0.22	0.50
Elastase	RGES	1.43	0.75	2.12
Glucosidase	DGLUC	0.94	0.42	1.46
Acid Phosphatase	DPO4	54.29	17.08	91.49
Galactidase	GALAC	0.38	0.00	1.05
Cathepsin C	TP8.7 M	30.99	16.24	45.75
Cathepsin C	TP8.7	35.01	24.75	45.26

TABLE 3A (continued)

ENZYME	SUBSTRATE	LYMPHS		
		MEAN	LO	HI
Gluc sidase	FL GLUC	0.23	0.15	0.32
Neutral Butyrate	DIBUT 7.5			
Acid Butyrate	DIBUT 6.5	4.08	0.00	12.16
Esterase	CLOAC	2.49	0.85	4.13
Esterase	DIACET 6.5	109.89	39.37	180.41
Esterase	DIACET 7.5	89.30	0.00	219.98
Acid Prop Esterase	DIPROP 6.5	86.11	0.00	216.25
Neutral Prop Esterase	DIPROP 7.5	96.34	0.00	219.08
Acidic Valerate Esterase	DIVAL 6.5	33.13	20.42	45.84
Acidic Hex Esterase	DIHEX 6.5	15.10	4.22	25.97
Neutral Hex Esterase	DIHEX 7.5	7.54	0.00	15.15
Acidic Hep Esterase	DIHEP 6.5	9.17	0.00	19.35
Neutral Hep Esterase	DIHEP 7.5	9.73	0.00	19.52
Acidic Pal Esterase	DIPAL 6.5	0.15	0.00	0.31
Neutral Pal Esterase	DIPAL 7.5	0.14	0.00	0.29

TABLE 3A   (continued)

NORMAL LEUKOCYTE ENZYME ACTIVITY				
			MONOS	
ENZYME	SUBSTRATE	MEAN	LO	HI
Aminopeptidase	LEU	120.66	35.52	205.80
Aminopeptidase M	ALA	172.33	107.51	237.15
Pro Aminopeptidase	PRO	6.83	2.62	11.04
Aminopeptidase M	LYS	7.56	3.83	11.29
Aminopeptidase M, N	GLY	61.47	30.13	92.82
Aminopeptidase N	SER	6.49	2.71	10.26
Endopeptidase I	ARG	16.80	7.83	25.78
Endopeptidase I	ARG-TFA	10.34	5.13	15.54
Aminopeptidase A	ASP	0.66	0.06	1.27
Cathepsin B	VS	19.61	2.86	36.36
Cathepsin B	VS-M	31.14	5.17	57.11
Cathepsin B	VK	20.38	0.00	54.92
Cathepsin B	VK-M	43.37	6.71	80.04
Cathepsin B	QS	10.66	4.02	17.30
Cathepsin B	QS-M	8.69	2.81	14.57
Cathepsin B	LG	44.56	22.50	56.62
Cathepsin B	LG-M	23.92	0.00	48.54
Dipeptidylpeptidase II	KA	39.35	16.82	61.88
Dipeptidylpeptidase II	KA-M	8.74	1.47	16.01
Dipeptidylpeptidase IV	Z-AA	328.25	243.23	413.27
Dipeptidylpeptidase IV	Z-AA-M	210.71	112.01	309.42
Dipeptidylpeptidase IV	Z-GP	225.10	155.83	294.37
Dipeptidylpeptidase IV	Z-GP-M	119.81	70.14	169.48
Cathepsin D	GL	41.78	20.02	63.54
Cathepsin D	GL-M	89.11	0.00	195.37
Cathepsin C	Z-AG	34.51	18.95	50.08
Cathepsin C	Z-AG-M	37.75	12.88	62.61
Dipeptidylpeptidase IV	AA-TFA	158.31	31.98	284.63
Dipeptidylpeptidase IV	AA-M	58.29	1.79	114.79
Cathepsin C	Z-TP6.5	49.69	22.25	77.13
Cathepsin C	Z-TP6.5M	45.70	21.61	69.80
Cathepsin B	LLR	54.32	28.31	80.32
Cathepsin B	LLR-M	62.85	14.37	111.33
Cathepsin B	LGLG	11.36	0.00	23.64
Cathepsin B	LGLG-M	40.74	0.00	98.16
Esterase	FDA	56.25	17.06	115.43
Monocytic Esterase	FDA-NAF	93.36	0.00	210.77
Peroxidase	DCFH-DAMES	12.28	2.23	22.34
Activated Peroxidase	DCHFMESPMA	22.18	0.00	48.35
Collagenase	GPLGP	23.38	10.98	35.79
Collagenase	GPLGP-M	23.32	10.36	36.28
Collagenase	GFGA	1.01	0.00	2.01
Elastase	RGES	8.37	4.28	12.46
Glucosidase	DGLUC	5.58	0.25	10.91
Acid Ph sphatase	DPO4	148.32	61.42	235.23
Galactidase	GALAC	5.94	0.00	17.90
Cathepsin C	TP8.7 M	71.45	38.94	103.97
Cathepsin C	TP8.7	76.00	53.89	98.11

TABLE 3A (continued)

ENZYME	SUBSTRATE	MONOS		
		MEAN	LO	HI
Gluc sidase	FL GLUC	1.89	0.00	4.55
Neutral Butyrate	DIBUT 7.5			
Acid Butyrate	DIBUT 6.5	10.37	0.00	30.20
Esterase	CLOAC	7.33	1.76	12.89
Esterase	DIACET 6.5	534.00	322.46	745.54
Esterase	DIACET 7.5	432.78	0.00	986.33
Acid Prop Esterase	DIPROP 6.5	441.65	0.00	1040.17
Neutral Prop Esterase	DIPROP 7.5	489.27	0.00	1106.82
Acidic Valerate Esterase	DIVAL 6.5	184.51	119.00	250.02
Acidic Hex Esterase	DIHEX 6.5	72.87	24.73	121.00
Neutral Hex Esterase	DIHEX 7.5	37.22	10.06	64.37
Acidic Hep Esterase	DIHEP 6.5	32.25	3.51	60.99
Neutral Hep Esterase	DIHEP 7.5	36.15	7.40	64.89
Acidic Pal Esterase	DIPAL 6.5	0.30	0.16	0.45
Neutral Pal Esterase	DIPAL 7.5	0.30	0.13	0.47

TABLE 3A (continued)

NORMAL LEUKOCYTE ENZYME ACTIVITY				
			GRANS	
ENZYME	SUBSTRATE	MEAN	LO	HI
Aminopeptidase	LEU	80.19	28.42	131.96
Aminopeptidase M	ALA	149.02	85.05	212.98
Pro Aminopeptidase	PRO	9.19	3.96	14.41
Aminopeptidase M	LYS	4.27	0.00	8.88
Aminopeptidase M, N	GLY	29.48	9.82	49.14
Aminopeptidase N	SER	3.40	0.75	6.06
Endopeptidase I	ARG	7.22	0.37	14.06
Endopeptidase I	ARG-TFA	5.16	0.23	10.10
Aminopeptidase A	ASP	0.45	0.01	0.90
Cathepsin B	VS	8.27	2.68	13.87
Cathepsin B	VS-M	7.07	1.75	12.40
Cathepsin B	VK	5.10	0.65	9.54
Cathepsin B	VK-M	4.31	0.00	8.76
Cathepsin B	QS	4.67	2.08	7.25
Cathepsin B	QS-M	2.71	1.43	4.00
Cathepsin B	LG	29.99	11.28	48.69
Cathepsin B	LG-M	10.54	3.83	17.26
Dipeptidylpeptidase II	KA	29.15	9.25	49.05
Dipeptidylpeptidase II	KA-M	1.74	0.82	2.65
Dipeptidylpeptidase IV	Z-AA	363.20	273.53	452.87
Dipeptidylpeptidase IV	Z-AA-M	252.24	138.76	365.72
Dipeptidylpeptidase IV	Z-GP	240.99	148.98	332.99
Dipeptidylpeptidase IV	Z-GP-M	130.62	63.36	197.87
Cathepsin D	GL	29.16	11.99	46.33
Cathepsin D	GL-M	12.01	0.00	27.36
Cathepsin C	Z-AG	33.19	17.20	49.18
Cathepsin C	Z-AG-M	34.70	9.31	60.09
Dipeptidylpeptidase IV	AA-TFA	154.81	25.75	283.87
Dipeptidylpeptidase IV	AA-M	29.91	9.29	50.53
Cathepsin C	Z-TP6.5	58.05	24.15	91.96
Cathepsin C	Z-TP6.5M	55.07	23.22	86.91
Cathepsin B	LLR	9.46	0.00	23.73
Cathepsin B	LLR-M	7.58	0.00	24.34
Cathepsin B	LGLG	4.08	1.93	6.23
Cathepsin B	LGLG-M	3.24	1.12	5.37
Esterase	FDA	68.37	12.90	123.84
Monocytic Esterase	FDA-NAF	81.99	17.39	146.60
Peroxidase	DCFH-DAMES	7.49	0.95	14.04
Activated Peroxidase	DCHFMESPMA	11.02	0.00	28.46
Collagenase	GPLGP	16.88	6.80	26.96
Collagenase	GPLGP-M	13.82	4.39	23.24
Collagenase	GFGA	1.00	0.36	1.63
Elastase	RGES	3.96	2.09	5.83
Glucosidase	DGLUC	2.35	0.10	4.60
Acid Phosphatase	DPO4	85.42	13.42	157.42
Galactidase	GALAC	1.58	0.00	4.32
Cathepsin C	TP8.7 M	80.16	45.97	114.35
Cathepsin C	TP8.7	82.35	59.36	105.33

TABLE 3A   (continued)

ENZYME	SUBSTRATE	GRANS		
		MEAN	LO	HI
Glucosidase	FL GLUC	0.45	0.07	0.83
Neutral Butyrate	DIBUT 7.5			
Acid Butyrate	DIBUT 6.5	6.43	0.00	18.24
Esterase	CLOAC	6.48	1.40	11.57
Esterase	DIACET 6.5	408.88	251.29	566.46
Esterase	DIACET 7.5	348.01	0.00	781.34
Acid Prop Esterase	DIPROP 6.5	349.18	0.00	838.78
Neutral Prop Esterase	DIPROP 7.5	429.17	0.00	977.31
Acidic Valerate Esterase	DIVAL 6.5	92.81	47.67	137.95
Acidic Hex Esterase	DIHEX 6.5	33.84	11.39	56.29
Neutral Hex Esterase	DIHEX 7.5	17.57	2.89	32.24
Acidic Hep Esterase	DIHEP 6.5	18.57	3.86	33.28
Neutral Hep Esterase	DIHEP 7.5	20.41	0.92	39.89
Acidic Pal Esterase	DIPAL 6.5	0.14	0.09	0.20
Neutral Pal Esterase	DIPAL 7.5	0.14	0.10	0.19

TABLE 3B

NORMAL ERYTHROCYTE ENZYME ACTIVITY					
SUBSTRATE	ENZYME		MEAN	2SD LO	2SD HI
LEU	Aminopeptidase		0.107	0.105	0.108
ALA	Aminopeptidase M		0.237	0.000	0.602
PRO	Pro Aminopeptidase		0.121	0.087	0.155
LYS	Aminopeptidase M		0.109	0.099	0.118
GLY	Aminopeptidase M, N		0.163	0.025	0.300
SER	Aminopeptidase N		0.107	0.107	0.107
ARG	Endopeptidase I		0.106	0.106	0.106
ARG-TFA	Endopeptidase I		0.106	0.104	0.107
ASP	Aminopeptidase A		0.106	0.106	0.106
VS	Cathepsin B		0.138	0.073	0.203
VS-M	Cathepsin B		0.176	0.027	0.324
VK	Cathepsin B		0.17	0.097	0.242
VK-M	Cathepsin B		0.129	0.093	0.164
QS	Cathepsin B		0.121	0.108	0.133
QS-M	Cathepsin B		0.116	0.113	0.119
LG	Cathepsin B		0.164	0.080	0.195
LG-M	Cathepsin B		0.131	0.129	0.132
KA	Dipeptidylpeptidase II		0.118	0.096	0.139
KA-M	Dipeptidylpeptidase II		0.11	0.104	0.116
Z-AA	Dipeptidylpeptidase IV		0.484	0.000	1.454
Z-AA-M	Dipeptidylpeptidase IV		0.411	0.000	1.227
Z-GP	Dipeptidylpeptidase IV		0.438	0.000	0.976
Z-GP-M	Dipeptidylpeptidase IV		0.334	0.000	0.818
GL	Cathepsin D		0.19	0.111	0.269
GL-M	Cathepsin D		0.286	0.000	0.734
Z-AG	Cathepsin C		0.225	0.000	0.482
Z-AG-M	Cathepsin C		0.171	0.115	0.226
AA-TFA	Dipeptidylpeptidase IV		0.144	0.070	0.218
AA-M	Dipeptidylpeptidase IV		0.131	0.097	0.165
Z-TP6.5	Cathepsin C		0.132	0.098	0.166
Z-TP6.5M	Cathepsin C		0.168	0.070	0.265
LLR	Cathepsin B		0.225	0.000	0.529
LLR-M	Cathepsin B		0.476	0.000	1.492
LGLG	Cathepsin B		0.122	0.091	0.153
LGLG-M	Cathepsin B		0.15	0.059	0.241
FDA	Esterase		0.126	0.089	0.163
FDA-NAF	Monocytic Esterase		0.136	0.088	0.184
DCFH-DAMES	Peroxidase		0.286	0.000	0.764
DCHFMESPMA	Activated Peroxidase		0.268	0.000	0.693
GPLGP	Collagenase		0.164	0.136	0.192
GPLGP-M	Collagenase		0.119	0.103	0.134
GFGA	Collagenase		0.131	0.073	0.188
RGES	Elastase		0.118	0.098	0.138
DGLUC	Glucosidase		0.114	0.109	0.118
DPO4	Acid Phosphatase		0.151	0.090	0.211
GALAC	Galactidase		0.119	0.111	0.127
TP8.7 M	Cathepsin C		0.212	0.000	0.467
TP8.7	Cathepsin C		0.161	0.054	0.267
FL GLUC	Glucosidase		0.11	0.110	0.110

TABLE 3C

NORMAL PLATELET ENZYME ACTIVITY					
SUBSTRATE	ENZYME		MEAN	2SD LO	2SD HI
LEU	Aminopeptidase		1.006	0.640	2.156
ALA	Aminopeptidase M		1.726	0.846	3.422
PRO	Pro Aminopeptidase		0.295	0.111	0.854
LYS	Aminopeptidase M		0.232	0.110	0.440
GLY	Aminopeptidase M, N		3.622	0.897	8.030
SER	Aminopeptidase N		0.309	0.135	0.579
ARG	Endopeptidase I		0.262	0.133	0.448
ARG-TFA	Endopeptidase I		0.268	0.128	0.499
ASP	Aminopeptidase A		0.403	0.170	1.274
VS	Cathepsin B		0.576	0.175	2.007
VS-M	Cathepsin B		0.592	0.180	1.622
VK	Cathepsin B		0.839	0.144	3.080
VK-M	Cathepsin B		0.486	0.153	1.343
QS	Cathepsin B		0.481	0.185	1.176
QS-M	Cathepsin B		0.499	0.280	1.194
LG	Cathepsin B		0.47	0.182	0.922
LG-M	Cathepsin B		0.438	0.143	1.032
KA	Dipeptidylpeptidase II		0.447	0.161	1.034
KA-M	Dipeptidylpeptidase II		0.277	0.126	0.562
Z-AA	Dipeptidylpeptidase IV		1.372	0.480	3.438
Z-AA-M	Dipeptidylpeptidase IV		1.199	0.545	3.253
Z-GP	Dipeptidylpeptidase IV		2.668	0.864	5.867
Z-GP-M	Dipeptidylpeptidase IV		1.415	0.481	3.883
GL	Cathepsin D		0.702	2.010	1.930
GL-M	Cathepsin D		0.511	0.254	1.235
Z-AG	Cathepsin C		7.9	0.411	21.322
Z-AG-M	Cathepsin C		5.113	0.280	13.295
AA-TFA	Dipeptidylpeptidase IV		0.622	0.218	1.274
AA-M	Dipeptidylpeptidase IV		0.35	0.145	0.684
Z-TP6.5	Cathepsin C		2.105	0.251	4.841
Z-TP6.5M	Cathepsin C		2.348	0.347	5.889
LLR	Cathepsin B		2.15	0.484	5.562
LLR-M	Cathepsin B		0.434	0.239	0.886
LGLG	Cathepsin B		2.275	0.230	10.086
LGLG-M	Cathepsin B		4.013	0.211	9.648
FDA	Esterase		15.35	2.960	29.955
FDA-NAF	Monocytic Esterase		7.201	2.310	14.369
DCFH-DAMES	Peroxidase		0.764	0.317	1.840
DCHFDAATRIS	Super oxide dismutase		3.556	1.640	7.982
DCHFMESPMA	Activated Peroxidase		1.979	0.576	7.045
DCFHMESPMA	Activated Super oxide dismutase		5.111	1.870	12.045

Artificial Intelligence By Back Propagation (Neural Net)

An alternate method of analyzing the data is via neural net.

To determine the interrelationship of enzyme function in both the cell and cell type, the ratios of enzyme activities needs to be analyzed. To fully analyze all possible combinations of a data set, an artificial intelligence system such as "Neuroshell™" (Ward Systems Group Inc., Frederick, MD) may be used.

The basic building block of artificial intelligence neural network technology is the simulated neuron, which processes a number of inputs to produce an output. Inputs and outputs are numeric values between 0 and 1 which represents positive stimulation close to 1 and negative stimulation close to 0. Inputs are data entered and outputs either come from other neurons or are displayed as results. The process by which the neuron processes its inputs to arrive at an output is usually a summation of inputs followed by a linear function applied to the sum. Independent neurons are of little use unless connected to a network of neurons called nodes. Nodes are layered and interconnected to receive information from each other. As each input node passes information to each other and the next layer, the values are weighed to represent the connection strength. To positively reinforce a connection the weight is raised and likewise to negatively reinforce or inhibit a connection the weight is lowered. The network processes data by accepting input patterns into input nodes or Defining Characteristics. The network produces output patterns which are called Classifying Characteristics. The user of the algorithm can adjust the output pattern by adjusting output thresholds. Feedback from the user determines whether the reinforcement is positive or negative. Learning in a neural network occurs when a set of input patterns (cell type and enzyme concentration) is given with a known

output pattern (Disease state or Normal). This is
called a sample case. The error between the predicted
and actual outputs for a given output node is measured
and the total error is one-half of the sum of the

5   squares of the difference. The weights leading to this
output node are modified slightly (specified by the user
as learning rate, a percentage of the error to be used
in the next iteration) during each iteration of a
learning session in the direction required to produce a

10   smaller error the next time the same pattern is
presented. This is how the neural network "learns".
Learning is continued until the consummate error of all
output nodes falls below a learning threshold controlled
by the user. Upon completion of learning, the network

15   should be capable of reproducing the correct output
pattern (disease or normal) when presented with one of
the input patterns it has learned. Moreover, the
network is capable of generalizing by recognizing an
input pattern close to a pattern it has learned and

20   produces an output close to a pattern it was trained to
produce. A simple two layered network is incapable of
learning complex patterns. Back propagation uses one or
more layers of hidden nodes and a nonlinear function
algorithm The weight applied to the nodes must then be

25   back propagated through all layers of the nodes. The
number of hidden nodes is determined by the user for a
specific problem. If too few hidden nodes are used,
then all the unique situations found in the sample case
will not be explored and if too many are used, learning

30   will never complete. The neural network approach
provides the opportunity to look at ratios of cell types
and enzyme levels in all possible combinations for both
disease and health using a simple format.

       Progression of a disease during treatment to

35   monitor "return to normalcy" or further increase in
stage or complication with additional disease states can
be done by monitoring the NNLS predictive disease

probabilities over time or the value of the Neural
Network score as it approaches normalcy or the three-
dimensional plotting of cell-type enzyme activity
patterns comparison to normal. Recurrent Neural
5 Networks may also be used for time series data.
Examples of these types are the Probabilistic Neural
Network (PNN), General Regression Neural Network (GRNN)
and the Kohonen-Realty Neural Network.

One embodiment of an analysis system using neural
10 networks is illustrated in Fig. 15. The same database
illustrated in Fig. 14A using the ANOVA technique is
also used in the neural network implementation. The
database is input into this neural network has a
training set and the user sets a threshold, learning
15 rate, and learning momentum. Once the neural network
has "learned" on the plurality of sample cases, a test
case is input to the neural network including an unknown
to be classified as a disease state or normal state.
Based on what the neural network as learned with a
20 plurality of sample cases, the neural network outputs
the best predictor of the test case. The neural network
may also be utilized to perform time course monitoring
of an individual patient for return to normalcy, using
an advanced probabilistic neural network (TNN) program.

25   **Look-Up Tables**
An alternative to the NNLS and Neural Net analyses
described above uses Look-Up tables and is similar to
the Expert System described in "White Cell and
Thrombocyte Disorders - Standardized Self-learning Flow
30 Cytometric List Mode Data Classification with the
CLASSIF1 Program System", Valet et al, Ann. N.Y. Acad.
Sci., 677:233-251 (1993).

Definitions

As used herein, either individually or as part of a larger group, "alkyl" means a linear, cyclic, or branched-chain aliphatic moiety of one to 10 carbon atoms; "substituted alkyl" means an alkyl group having a substituent containing a heteroatom or heteroatoms such as N, O, or S; "aryl" means an aromatic moiety, e.g., phenyl, of 6 to 18 carbon atoms, unsubstituted or substituted with one or more alkyl, substituted alkyl, nitro, alkoxy, or halo groups; and "alkaryl" means an aryl moiety of 7 to 19 carbons having an aliphatic substituent, and optionally, other substituents such as one or more alkyl, substituted alkyl, alkoxy or amino groups. "Aralkyl" means a linear or branched-chain aliphatic moiety of six to 18 carbon atoms comprising an aryl group or groups.

The following common chemical abbreviations are used in the examples:

```
t-BOC = tertiarybutyloxycarbonyl
EDAC = 1-ethyl-3-(3'-dimethylaminopropyl-
       carbodiimide)-hydrochloride
FMOC = 9-fluorenylmethyloxycarbonyl
BOP  = benzotriazoly-N-oxy-tris(dimethylamino)-
       phosphonium-hexafluorophosphate
HBOT = 1-hydroxybenzotriazole
HPLC = High pressure liquid chromatography
TLC  = Thin layer chromatography
V:V  = Volume to volume
```

The amino acids are abbreviated as follows:

Amino Acid	Abbreviation
L-alanine	Ala or A
L-arginine	Arg or R
L-asparagine	Asn or N
L-aspartic acid	Asp or D
L-cysteine	Cys or C
L-glutamic acid	Glu or E
L-glutamine	Gln or Q
glycine	Gly or G
L-histidine	His or H
L-isoleucine	Ile or I

L-leucine	Leu or L
L-lysine	Lys or K
L-methionine	Met or M
L-phenylalanine	Phe or F
L-proline	Pro or P
L-serine	Ser or S
L-threonine	Thr or T
L-tryptophan	Trp or W
L-tyrosine	Tyr or X
L-valine	Val or V

The synthesis of the assay compounds can be further understood by reference to the Examples. It will be appreciated, however, that the invention is not limited to the described examples, and that other methods of preparation could be suitable to prepare reagents according to the invention.

Example 1:

Preparation of Monopeptide Derivative of Rhodamine 110 Employing the EDAC Procedure

A 10-fold excess of a FMOC amino acid is placed into a round bottom flask containing a 50:50 pyridine-dimethylformamide solution (V:V) and stirred until a complete solution occurs. To this stirred solution is added a 12-fold excess of EDAC and the admixture is stirred for 30 minutes. A solution of rhodamine 110 dissolved in a minimum of a 50:50 pyridine-dimethylformamide (V:V) is added dropwise to the reaction solution. This addition requires 15-20 minutes and the reaction solution is allowed to stir at room temperature overnight. The solution is concentrated under reduced pressure to an oil. This oil is dissolved into an appropriated organic solvent and the product is purified by normal phase HPLC, using solvents of increasing polarity (methylene chloride, 1% methanol-chloroform, 2% methanol-chloroform, etc.). The eluate

containing the product is concentrated under reduced
pressure affording a crystalline material and the purity
and identity are checked by analytical reverse phase
high pressure liquid chromatography and thin layer
5   chromatography.

The crystalline material is treated with a 5%
solution of piperidine dissolved in dimethylformamide.
The reaction is stirred for 45 minutes and concentrated
under reduced pressure.   The resulting solid is
10   triturated several times with pentane and then dissolved
in a minimum of methanol and a 5-fold excess of
trifluoroacetic acid is added.   The solution is
concentrated under reduced pressure to dryness and the
resulting solid is centrifuged with cold diethyl ether
15   until the ether triturate has a pH = 7.   If the
monopeptide is polar, then the remaining protective
group is removed by treating with a 30 to 50%
trifluoroacetic acid solution in methylene chloride for
four hours at room temperature.   The solution is
20   concentrated under reduced pressure to dryness and the
resulting solid is centrifuged with cold diethyl ether
until the ether triturate has a pH = 7.   A final
purification of this trifluoroacetic acid substrate is
effected with reverse phase HPLC, using solvents of
25   decreasing   polarity   (water,   acetonitrile,
trifluoroacetic acid).   The eluate containing the
product is concentrated under reduced pressure and the
aqueous solution is lyophilized. The product's purity
and identity are checked by analytical reverse phase
30   high pressure liquid chromatography, thin layer
chromatography, and photon counting spectrofluorometry.
The purity and stability of the product are also
measured by monitoring the background fluorescence,
autohydrolysis and enzymatic activity using the product
35   as a substrate after storage of the product at 4°C.
Figs. 10A and 10B illustrate the stability and purity of
a monopeptide-TFA salt derivative Proline-rhodamine 110

which was prepared by the procedure described in this
Example. Stability (background fluorescence) is shown
in Fig. 10A. Autohydrolysis (diamonds) and enzyme rate
(squares) are shown in Fig. 10B.

Example 2:

Preparation of Dipeptide Derivative of Rhodamine 110 Employing the EDAC Procedure

A 6-fold excess of the FMOC amino acid is placed
into a round bottom flask containing a 50:50 pyridine-
dimethylformamide solution (V:V) and stirred several
minutes. To this well-stirred solution is added a 12-
fold excess of EDAC and the admixture is stirred an
additional 30 minutes. A solution of the monopeptide of
rhodamine 110 dissolved in a minimum of 50:50 pyridine-
dimethylformamide solution (V:V) is added dropwise over
a period of 15 to 20 minutes. The reaction is stirred
at room temperature for 16 hours and then concentrated
to an oil under reduced pressure. This oil is dissolved
in a minimum of an organic solvent and the crude product
is purified by normal phase HPLC. The eluate containing
the desired product is collected and concentrated under
reduced pressure affording a crystalline material and
the purity and identity are checked by analytical
reverse phase high pressure liquid chromatography and
thin layer chromatography. The FMOC blocking group is
removed by dissolving the solid in a 5% piperidine-
dimethylformamide solution and stirred at room
temperature for one hour. The solution is concentrated
to dryness under reduced pressure. The resulting solid
is triturated several times with pentane to remove the
FMOC polymer. The solid is dissolved in a minimum of
methanol and a 5-fold excess of trifluoroacetic acid is
added. The solution is concentrated to dryness and the
resulting solid is centrifuged with cold diethyl ether
until the ether triturate has a pH = 7. If the
dipeptide is polar, then the remaining protective
gr up(s) is remov d by treating with a 30 to 50%

trifluoroacetic acid solution in methylene chloride for
four hours at room temperature.   The solution is
concentrated under reduced pressure to dryness and the
resulting solid is centrifuged with cold diethyl ether
5   until the ether triturate has a pH = 7.   A final
purification of this trifluoroacetic acid substrate is
effected with reverse phase HPLC. The eluate containing
the product is concentrated under reduced pressure and
the aqueous solution is lyophilized.   The product's
10   purity and identity are checked by analytical reverse
phase high pressure liquid chromatography, thin layer
chromatography and photon counting spectrofluorometry.
The purity and stability of the product are also
measured by monitoring the background fluorescence,
15   autohydrolysis and enzymatic activity using the product
as a substrate after storage of the product at 4°C.
Figs. 11A-11F illustrate the stability and purity of the
TFA salts of several dipeptide derivatives of rhodamine
110 prepared by the procedure described in this Example.
20   The Figures describe the following:
         Fig. 11A (background fluorescence, Val-Lys•TFA);
         Fig. 11B (autohydrolysis and enzyme rate, Val-
Lys•TFA);
         Fig. 11C (background fluorescence, Val-Ser•TFA);
25   Fig. 11D (autohydrolysis and enzyme rate, Val-
Ser•TFA);
         Fig. 11E (background fluorescence, Leu-Gly•TFA);
and
         Fig. 11F (autohydrolysis and enzyme rate, Leu-
30   Gly•TFA).

Example 3:

Preparation of Polypeptide Derivative of
Rhodamine 110 Employing the EDAC Procedure

         A 6-fold excess of the FMOC polyamino acid is
35   placed into a round bottom flask containing a 50:50
pyridine-dimethylformamide solution (V:V) and stirred

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until solution occurs. To this well stirred solution is
added a 12-fold excess of EDAC and the admixture is
stirred an additional 30 minutes. A solution of the
monopeptide of rhodamine 110 dissolved in a minimum of
5   50:50 pyridine-dimethylformamide solution (V:V) is added
dropwise over a period of 15 to 20 minutes. The
reaction is stirred at room temperature for 16 hours and
then concentrated to an oil under reduced pressure. The
oil is dissolved in a minimum of an organic solvent and
10   the crude product is purified by normal phase HPLC. The
eluate containing the desired product is collected and
concentrated under reduced pressure affording a
crystalline material and the purity and identity are
checked by analytical reverse phase high pressure liquid
15   chromatography and thin layer chromatography. The FMOC
blocking group is removed by dissolving the solid in a
5% piperidine dimethylformamide solution and stirred at
room temperature for one hour. The solution is
concentrated under reduced pressure to dryness under
20   reduced pressure. The resulting solid is triturated
several times with pentane to remove the FMOC polymer.
The solid is dissolved in a minimum of methanol and a 5-
fold excess of trifluoroacetic acid is added. The
solution is concentrated under reduced pressure to
25   dryness and the resulting solid is centrifuged with cold
diethyl ether until the ether triturate has a pH = 7.
If the polypeptide is polar then the remaining group(s)
is removed by treating with a 30 to 50% trifluoroacetic
acid solution for four hours at room temperature. The
30   solution is concentrated under reduced pressure to
dryness, and the resulting solid is centrifuged with
cold diethyl ether until the ether triturate has a pH =
7. A final purification of this trifluoroacetic acid
substrate is effected with reverse phase HPLC. The
35   eluate containing the product is concentrated under
reduced pressure and the aqueous solution is
lyophilized. The product's purity and identity are

checked by analytical reverse phase high pressure liquid
chromatography, thin layer chromatography and photon
counting spectrofluorometry.

Example 4:
Preparation of a Dipeptide Derivative of
Rhodamine 110 Employing the HOBT-BOP Procedure

5

A 4-fold excess of the FMOC amino acid, and a 4-
fold excess of HOBT and BOP are placed into a round
bottom flask containing a 0.6 millimolar solution of N-
10   methylmorpholine in dimethylformamide and stirred for
10-15 minutes. To this solution is added dropwise a
solution of the monopeptide of rhodamine 110 dissolved
in a minimum amount of a 0.6 millimolar solution of N-
methylmorpholine in dimethylformamide. This addition
15   requires 5-10 minutes, and the reaction is stirred at
room temperature for four hours. The reaction solution
is concentrated under reduced pressure to an oil. This
oil is dissolved in methylene chloride and the crude
product is purified by normal phase HPLC. The eluate
20   containing the desired product is collected and
concentrated under reduced pressure affording a
crystalline material. The purity and identify of this
material are checked by analytical reverse phase HPLC
and thin layer chromatography. The FMOC blocking is
25   removed by dissolving the solid in a 5% piperidine-
dimethylformamide solution and stirred at room
temperature for one hour. The solution is concentrated
under reduced pressure, and the resulting solid is
triturated several times with pentane to remove the FMOC
30   polymer. The remaining solid is dissolved in a minimum
of methanol and a 5-fold excess of trifluoroacetic acid
is added. The solution is concentrated under reduced
pressure and the resulting solid is centrifuged with
cold diethyl ether until the ether triturate has a pH =
35   7. If the dipeptide is polar then the remaining
protective group(s) is removed by treating with a 30 to

50% trifluoroacetic acid solution in methylene chloride
for four hours at room temperature. The solution is
concentrated under reduced pressure to dryness, and the
resulting solid is centrifuged with cold diethyl ether
5   until the ether triturate has a pH = 7.   A final
purification of this trifluoroacetic acid substrate is
effected with reverse phase HPLC. The eluate containing
the product is concentrated under reduced pressure and
the aqueous solution is lyophilized.   The product's
10   purity and identity are checked by analytical reverse
phase high pressure liquid chromatography, thin layer
chromatography and photon counting spectrofluorometry.

Example 5:

Preparation of a Polypeptide Derivative of
15   **Rhodamine 110 Employing the HOBT-BOP Procedure**

A 4-fold excess of the FMOC polypeptide and a 4-
fold excess of HOBT and BOP are placed into a round
bottom flask containing a 0.6 millimolar solution of N-
methylmorpholine in dimethylformamide and stirred for
20   10-15 minutes. To this solution is added dropwise a
solution of the monopeptide rhodamine 110 dissolved in
a minimum amount of a 0.6 millimolar solution of N-
methylmorpholine in dimethylformamide. This addition
requires 5-10 minutes, and the reaction is stirred at
25   room temperature for four hours. The reaction solution
is concentrated under reduced pressure to an oil. This
oil is dissolved in methylene chloride and the crude
product is purified by normal phase HPLC. The eluate
containing the desired product is collected and
30   concentrated   under   reduced   pressure   affording   a
crystalline material. The purity and identify of this
material are checked by analytical reverse phase HPLC
and thin layer chromatography. The FMOC blocking is
removed by dissolving the solid in a 5% piperidine-
35   dimethylformamide   solution   and   stirred   at   room
temperature for one hour. The solution is concentrated
under reduced pressure, and the resulting solid is

triturated several times with pentane to remove the FMOC
polymer. The remaining solid is dissolved in a minimum
of methanol and a 5-fold excess of trifluoroacetic acid
is added. The solution is concentrated under reduced
5   pressure and the resulting solid is centrifuged with
cold diethyl ether until the ether triturate has a pH =
7. If the polypeptide is polar then the remaining
protective group(s) is removed by treating with a 30 to
50% trifluoroacetic acid solution in methylene chloride
10   for four hours at room temperature. The solution is
concentrated under reduced pressure to dryness, and the
resulting solid is centrifuged with cold diethyl ether
until the ether triturate has a pH = 7. A final
purification of this trifluoroacetic acid substrate is
15   effected with reverse phase HPLC. The eluate containing
the product is concentrated under reduced pressure and
the aqueous solution is lyophilized. The product's
purity and identify are checked by analytical reverse
phase HPLC, thin layer chromatography and photon
20   counting spectrofluorometry.

Example 6:
Preparation of p-Aminobenzoic Acid
Derivative of Rhodamine 110

A molar quantity of p-Aminobenzoic Acid is placed
25   into a round bottom flask containing a small quantity of
dioxane and stirred until a complete solution occurs.
A 10% molar excess solution of sodium carbonate,
dissolved in water, is added. To this well-stirred
solution is added dropwise a molar solution of 9-
30   fluorenylmethyloxycarbonylchloride dissolved in a
minimum of dioxane. This addition requires 10 to 15
minutes and the reaction solution is allowed to stir an
additional four (4) hours. The reaction is diluted with
water and extracted (3) times with diethyl ether. The
35   aqueous layer is cooled in an ice water bath and the pH
is adjusted to two (2) with a 10% solution of

hydrochloric acid. The resulting colorless precipitate is filtered and recrystallized from an acetone solution. A TLC of the colorless, crystalline product showed only one (1) quenched spot and obtained in a yield of 68%.

A 6-fold excess of the FMOC-p-amino acid is placed into a round bottom flask containing a 50:50 pyridine-dimethylformamide solution (V:V) and stirred until a complete solution occurs. To this stirred solution is added a 12-fold excess of EDAC and the admixture is stirred for 30 minutes. A solution of rhodamine 110, dissolved in a minimum of a 50:50 pyridine-dimethylformamide (V:V) is added dropwise to the reaction solution. This addition requires 15-20 minutes, and the reaction solution is concentrated under reduced pressure to an oil and dissolved in a small amount of chloroform. The crude product is purified by normal phase HPLC and the product is eluted in a 5% methanol-chloroform solution. This eluate is concentrated under reduced pressure, and the resulting colorless solid dried in vacuo affording a 60% yield of the product. This material is treated with a 5% solution of piperidine dissolved in dimethylformamide. The resulting solution is stirred at room temperature for one (1) hour and concentrated under reduced pressure. The resulting solid is triturated several times with pentane, dissolved in a minimum of methanol and a 5-fold excess of trifluoroacetic acid is added. The solution is concentrated to dryness and the resulting solid is centrifuged with cold diethyl ether until the ether triturate has a pH of seven (7). The trifluoroacetate salt is dried in vacuo overnight affording a yield of 74.5%. The product's purity and identify are checked by analytical reverse phase high pressure liquid chromatography, thin layer chromatography and photon counting spectrofluorometry.

Example 7:

Preparation of Tetraacetyl-α-D-Glucopyranosyl Derivative of Rhodamine 110 and Tetrabenzoyl-α-D-Glucopyranosyl Derivative of Rhodamine 110

5   A 10-fold excess of the respective protected tetraacetyl (or tetrabenzoyl) α-D-glucopyranosyl bromides is placed into a round bottom flask containing a 50:50 pyridine-dimethylformamide solution (V:V) and warmed and stirred until a complete solution occurs. To
10   this stirred solution is added a 12-fold excess of EDAC and the admixture is stirred for 30 minutes. A solution of rhodamine 110, dissolved in a minimum of a 50:50 pyridine-dimethylformamide (V:V) is added dropwise to the reaction solution. This addition requires 15 to 20
15   minutes. The reaction solution is allowed to stir for 24 hours and concentrated under reduced pressure to an oil. The crude product is dissolved in chloroform and purified by normal phase HPLC. The tetraacetyl-α-D-glucopyranosyl derivative is eluted in a 1% methanol-
20   chloroform solution. The tetrabenzoyl-α-D-glucopyranosyl derivative is eluted in a 3% methanol-chloroform solution. The product is isolated by concentrating the respective eluates under reduced pressure and drying in vacuo overnight. The yield of
25   the tetraacetyl-α-D-glucopyranosyl rhodamine 110 is 30% and the yield of the tetrabenzoyl-α-D-glucopyranosyl rhodamine 110 is almost quantitative (100%). The product's purity and identity are checked by analytical reverse phase high pressure liquid chromatography, thin
30   layer chromatography and photon counting spectrofluorometry.

Example 8:
Preparation f N-Butyl
Ester Derivative of Fluorescein

A 3.4 fold excess of n-butyrylanhydride is placed
into a round bottom flask containing a minimum amount of
tetrahydrofuran and stirred for several minutes. A one
molar equivalent of fluorescein is added, followed by 10
mL of triethylamine and solution occurs. The reaction
solution is stirred an additional 30 minutes and then
concentrated under reduced pressure to an oil. The oil
is dissolved in a minimum of chloroform and the crude
product is purified by normal phase HPLC. The product
is eluted with a 0.5% methanol-chloroform solution and
the eluate is concentrated under reduced pressure
affording a yield of a colorless solid of 63%. The
product's purity and identity are checked by analytical
reverse phase high pressure liquid chromatography, thin
layer chromatography and photon counting
spectrofluorometry.

Example 9:
Preparation of Chloroacetyl
Ester Derivative of Fluorescein

A 10-fold excess of chloroacetic anhydride is
placed into a round bottom flask containing a minimum
amount of tetrahydrofuran and stirred for several
minutes. A solution of fluorescein, dissolved in a
minimum amount of tetrahydrofuran and a 2-fold excess of
triethylamine, is added dropwise to the reaction
mixture. This addition required 10 to 15 minutes and the
reaction solution is allowed to stir overnight. The
solution is concentrated under reduced pressure to an
oil. This oil is dissolved in a minimum amount of
methylene chloride and the crude product is purified by
normal phase HPLC. The desired product is eluted in a
100% methylene chloride solution, and this eluate
concentrated under reduced pressure. This solid is

dried in vacuo for 16 hours affording a quantitative yield (100%) of the desired product. The product's purity and identity are checked by analytical reverse phase high pressure liquid chromatography, thin layer

5   chromatography and photon counting spectrofluorometry.

Example 10:
Preparation of n-Palmityl
Ester Derivative of Fluorescein

A 2.5-fold excess of palmitic acid is placed into

10   a round bottom flask containing a minimum amount of tetrahydrofuran and stirred for several minutes. To this solution is added a 3-fold excess of EDAC and the mixture stirred for 30 minutes. A solution of fluorescein dissolved in a minimum amount of

15   tetrahydrofuran is added dropwise to the reaction mixture. This addition required 15 to 20 minutes and the reaction mixture is allowed to stir overnight. The reaction mixture is concentrated under reduced pressure to an oil. This oil is dissolved in chloroform and

20   extracted three (3) times with a 5% aqueous sodium bicarbonate solution. The organic layer is dried over magnesium sulfate, filtered and concentrated to dryness. This crude product is purified by normal phase HPLC and the product is eluted with 100% chloroform. This eluate

25   is concentrated under reduced pressure and the resulting solid is dried in vacuo affording 400 mg (9% yield). The product's purity and identity are checked by analytical reverse phase high pressure liquid chromatography, thin layer chromatography and photon

30   counting spectrofluorometry.

An elemental analysis for carbon and hydrogen, by Galbraith Laboratories, Inc. of Knoxville, TN, gave the following results: Formula: $C_{52}H_{72}O_7 \cdot \frac{1}{2}H_2O$

MW = 817.075

<u>Theoretical</u>	<u>Found</u>
C = 76.34	C = 76.36; 76.51
H = 8.99	H = 9.01; 9.05

Example 11:

Preparation of Diphenylphosphate Derivative of Rhodamine 110

A 6.6-fold excess of diphenylchlorophosphate is placed into a round bottom flask containing a very small amount of pyridine and stirred several minutes in an ice-water bath. To this well-stirred, cold solution is added rhodamine 110 and a white precipitate is formed immediately. The stirring is continued for an hour and the reaction mixture is placed in the refrigerator for 48 hours. The reaction mixture is treated with water and extracted twice with chloroform. The combined chloroform extracts are dried over magnesium sulfate, filtered and concentrated under reduced pressure. The crude product is purified by normal phase HPLC. The desired product is eluted in a 1% methanol-chloroform solution, and this eluate is concentrated under reduced pressure to an oil. This oil is dissolved in ammonia, and the resulting aqueous solution is lyophilized affording the ammonium salt of the product in a 66% yield. The product's purity and identity are checked by analytical reverse phase high pressure liquid chromatography, thin layer chromatography and photon counting spectrofluorometry.

Example 12:

Preparation of Diphenylphosphate
Derivative of Fluorescein

A 6.6-fold excess of diphenylchlorophosphate is
placed into a round bottom flask containing a very small
amount of pyridine and stirred several minutes in an
ice-water bath. To this well-stirred, cold solution is
added fluorescein and a white precipitate is formed
immediately. Stirring is continued for one (1) hour and
the reaction mixture placed in the refrigerator for 48
hours. The reaction mixture is treated with water and
extracted twice with chloroform. The combined
chloroform extracts are dried over magnesium sulfate,
filtered and concentrated to dryness under reduced
pressure. The resulting solid is dissolved in a minimum
amount of chloroform and the crude product is purified
by normal phase HPLC. The product is eluted in a 1%
methanol-chloroform solution and this solution
concentrated under reduced pressure. The colorless
solid is treated with ammonia and lyophilized, affording
the ammonium salt in a yield of 95%. The product's
purity and identity are checked by analytical reverse
phase high pressure liquid chromatography, thin layer
chromatography and photon counting spectrofluorometry.

Example 13:

Preparation of Trifluoroacetyl Ester
Derivative of 4'(5')Carboxyfluorescein

A 10-fold excess of trifluoroacetic anhydride is
placed into a round bottom flask containing a minimum
amount of tetrahydrofuran and stirred several minutes.
A 30% pyridine-tetrahydrofuran solution, containing the
4'(5')carboxyfluorescein, is added dropwise over 10 to
15 minutes. The solution is allowed to stir overnight
and concentrated under reduced pressure. The resulting
oil is dissolved in chloroform, extracted three times
with water and the organic layer dried over magnesium

sulfate. This is filtered, concentrated to a small volume under reduced pressure and the crude product purified by normal phase HPLC. The product is eluted in a 4% methanol-chloroform solution. This is concentrated under reduced pressure and resulting solid dried in vacuo for 15 hours affording an 83% yield. The product's purity and identity are checked by analytical reverse phase high pressure liquid chromatography, thin layer chromatography and photon counting spectrofluorometry.

Example 14:
Preparation of Diphenylphosphate
Ester Derivative of 4'(5')Carboxyfluorescein

A 15-fold excess of chlorodiphenylphosphate is added to a solution of 4'(5')carboxyfluorescein, dissolved in 8 mL of pyridine over a period of 10 to 15 minutes. The reddish-colored solution turns a light yellow and a precipitate is formed. Stirring is continued for two hours and the mixture is allowed to cool in the refrigerator overnight. To this mixture is added 100 mL of water and the mixture extracted three times with chloroform. The combined chloroform extracts are dried over magnesium sulfate, filtered and concentrated under reduced pressure to an oil. This oil is dissolved in a minimum amount of methylene chloride, and the crude product is purified by normal phase HPLC. The desired product is eluted in a 1% methanol-chloroform solution, and this eluate is concentrated under reduced pressure. The resulting solid is dried in vacuo for 16 hours affording a yield of 95%. The product's purity and identity are checked by analytical reverse phase high pressure liquid chromatography, thin layer chromatography and photon counting spectrofluorometry.

Exampl 15:

Preparation of H-L-Leucine Trifluoroacetate
Salt Derivative of Rhodol

A 10-fold excess of benzyloxycarbonyl-L-leucine is

5   placed into a round bottom flask containing a 50:50
pyridine-dimethylformamide solution (V:V) and stirred
until a complete solution occurs. To this stirred
solution is added a 12-fold excess of EDAC and the
admixture is stirred for 30 minutes. A solution of

10   rhodol hydrochloride, dissolved in a minimum of a
pyridine-dimethylformamide solution (V:V), is added
dropwise to the reaction solution. This addition
required 10 to 15 minutes and the reaction is allowed to
stir at room temperature overnight The solution is

15   concentrated under reduced pressure to an oil. This oil
is dissolved into chloroform and extracted three (3)
times with water and organic layer dried over magnesium
sulfate. This is filtered, concentrated to a very small
volume under reduced pressure and purified by normal

20   phase HPLC. The product is eluted in a 2% methanol-
chloroform solution. This eluate is concentrated under
reduced pressure and the resulting colorless,
crystalline solid, dried in vacuo, affords a 33.4% yield
of the product and the product's purity and identity are

25   checked by analytical reverse phase high pressure liquid
chromatography and thin layer chromatography. This
material is dissolved into a small volume of isopropyl
alcohol and catalytically reduced with a small amount of
10% palladium on carbon as the catalyst in a Paar shaker

30   apparatus for 16 hours. The alcohol solution is
carefully filtered and a 2-fold excess of
trifluoroacetic acid is added. This solution is
concentrated to dryness under reduced pressure and the
resulting solid is centrifuged with cold diethyl ether

35   until the ether triturate has a pH of 7. The colorless
trifluoroacetate salt is dried in vacuo overnight,
affording a 92.48% yield. The product's purity and

identity are checked by analytical reverse phase high
pressure liquid chromatography, thin layer
chromatography and photon counting spectrofluorometry.

Example 16:
Preparation of (H-LEU-GLY)₂
Rhodamine 110 Acetate and Tartrate Salts

A 10-fold excess of FMOC glycine is placed into a
round bottom flask containing a 50:50 pyridine-
dimethylformamide solution (v:v) and stirred until a
complete solution occurs. To this stirred solution is
added a 12-fold excess of EDAC and the admixture is
stirred for 30 minutes. A solution of rhodamine 110 is
dissolved in a minimum of a 50:50 pyridine-
dimethylformamide solution (v:v) and is added dropwise
to the reaction solution. This addition requires 15-20
minutes, and the reaction is allowed to stir at room
temperature overnight. The solution is concentrated
under reduced pressure to an oil. This oil is dissolved
in a small amount of methylene chloride, and the product
is purified by normal phase HPLC. The product is eluted
from the column in a 1% methanol-chloroform solution.
This eluate is concentrated under reduced pressure, and
the resulting solid dried in vacuo affording an 85%
yield of the product. The purity and identity are
checked by analytical reverse phase high pressure liquid
chromatography and thin layer chromatography. This
material is treated with a 5% solution of piperidine
dissolved in dimethylformamide. The resulting solution
is stirred at room temperature for one (1) hour and
concentrated under reduced pressure. The resulting
solid is triturated several times with pentane and
product dried in vacuo. A TLC of this material showed
only one quenched spot which is positive to concentrated
hydrochloric acid.

A four (4) fold excess of FMOC-L-leucine is placed
into a round bottom flask containing a 50:50 pyridine-
dimethylformamide solution (v:v) and stirred until a
complete solution occurs. To this stirred solution is

5   added an eight (8) fold excess of EDAC and the admixture
is stirred for 30 minutes. A solution of (H-GLY)$_2$
rhodamine 110 (from above) dissolved in a minimum of a
50:50 pyridine-dimethylformamide solution (v:v) is added
dropwise to the reaction solution. This addition

10 requires 15-20 minutes, and the reaction is allowed to
stir at room temperature for six (6) hours. The
solution is concentrated under reduced pressure to an
oil. This oil is dissolved in chloroform, and the
product is purified by normal phase high pressure liquid

15 chromatography. The product is eluted from the column
in a 2% methanol-chloroform solution. This eluate is
concentrated under reduced pressure, and the resulting
solid dried in vacuo affording a 74% yield. The purity
and identity are checked by analytical reverse phase

20 high pressure liquid chromatography and thin layer
chromatography. This material is treated with a 5%
solution of piperidine dissolved in dimethylformamide.
The resulting solution is stirred at room temperature
for one (1) hour and concentrated under reduced

25 pressure. The solid is triturated several times with
pentane and dried in vacuo. One half of this material
is dissolved in a small amount of methanol and a 10%
excess of acetic acid is added. Ether is added to this
solution and cooled in an ice-water bath. The resulting

30 colorless solid is filtered, washed with ether and
centrifuged with ether until the pH = 7. The
crystalline salt is dried in vacuo according a 61%
yield.

The remaining one-half of the material (from above)

35 is dissolved in a small amount of methanol and a 10%
excess of L-tartaric acid dissolved in a very small
amount of methanol is added. This solution is cooled in

an ice-water bath and ether is added. The resulting
crystalline material is filtered, washed with ether and
centrifuged with ether until the pH = 7. The resulting
colorless salt is dried in vacuo affording a 42% yield.

5   The free fluorescence and identity of the acetate and
tartrate salts of these rhodamine 110 substrates are
checked by analytical reverse phase high pressure liquid
chromatography, thin layer chromatography and photon
counting spectrofluorometry.

10   The acetate and tartrate salts thus prepared have
the following characteristics, respectively: native
free   fluorescence,   63,000   and   61,000   photons;
autohydrolysis rate when measured at 37°C using a 1 cm
path length, -5.56 and -3.8 change in photons per
15   second; and enzymatic reaction rate of cathepsin B at 37
°C, +128 and +138 change in photons per second. The
purity and stability of acetate and tartrate salts of
(LeuGly)$_2$ rhodamine 110 as demonstrated by assessment of
autohydrolysis, background fluorescence and enzymatic
20   activity after storage at 4°C are illustrated in Figs.
12A-12D as follows:

        Fig.   12A   (background   fluorescence,   Leu-
Gly•acetate);

        Fig. 12B (autohydrolysis and enzyme rate, Leu-
25   Gly•acetate);

        Fig.   12C   (background   fluorescence,   Leu-
Gly•tartrate); and

        Fig. 12D (autohydrolysis and enzyme rate, Leu-
Gly•tartrate).

30   Example 17:

        Preparation of the Free Amine of (Lys-Ala)$_2$
                       Rhodamine 110

        A 10-fold excess of the FMOC L-lysineεBOC amino
acid is placed into a round bottom flask containing a
35   50:50 pyridine-dimethylformamide solution (V:V) and
stirred several minutes. To this well-stirred solution
is added a 20-fold excess of EDAC and the admixture is

stirred an additional 30 minutes. A solution of (Ala)$_2$ rhodamine 110 dissolved in a minimum of 50:50 pyridine-dimethylformamide solution (V:V) is added dropwise over a period of 15 to 20 minutes. The reaction is stirred

5   at room temperature for 16 hours and then concentrated to an oil under reduced pressure. This oil is dissolved in a minimum of an organic solvent and the crude product is purified by normal phase HPLC. The eluate containing the desired product is collected and concentrated under

10   reduced pressure affording a crystalline material. A TLC of this material is run to check for purity and identity. The BOC protecting group is removed by dissolving the solid into a 50% solution of trifluoroacetic acid in methylene chloride. The

15   reaction is stirred at room temperature for one hour, and the purity of the reaction product is checked by thin layer chromatography. The TLC did not show any of the BOC group. The acid solution is concentrated under reduced pressure to dryness. Several washes with fresh

20   methylene chloride and reconcentrations under reduced pressure are performed to generate a crystalline solid. The FMOC blocking group is removed by dissolving the solid in a 5% piperidine-dimethylformamide solution and stirred at room temperature for one hour. The solution

25   is concentrated to dryness under reduced pressure. The resulting solid is triturated several times with pentane to remove the FMOC polymer and the product is dried in vacuo to constant weight affording a yield of 98.62%. The purity of this material is checked by reverse phase

30   HPLC, thin-layer chromatography and photon counting spectrofluorometry. The stability and purity of the product is further determined by monitoring the autohydrolysis, background fluorescence and enzymatic activity with the product as a substrate after storage

35   of the product at 4°C. Figs. 13A and 13B illustrate the stability of the free amine of (Lys-Ala)$_2$ rhodamine 110 which was prepared by the procedure described in this

Example.   Fig. 13A shows background fluorescence and Fig. 13B shows autohydrolysis and enzyme rate.

Example 18:

Use of Different Salts to Enhance Specificity

5   The use of salts to identify cellular enzymes is very important. pH optimas are different demonstrating different enzymes or isoenzymes. Different salts from within the same pH range may give different reactivities.   Z-groups, which are not salts but 10   covalent organic compounds, show relatively little activity and no pH optima. See Figs. 2A-2D, using cathepsin B as a target enzyme.

Example 19:

Use of Inhibitors in the Reagent Formula

15   Use of inhibitors of the targeted enzyme has been shown to prove substrate specificity.   More specifically, when an inhibitor eliminated the targeted enzyme signal, it was reasoned that the targeted enzyme activity was measured without the inhibitor.   The 20   disclosed enzyme assay contemplates the use of interfering reaction inhibitors to increase and maintain specificity.

To improve a Cathepsin D response, inhibitors to aminopeptidase and Cathepsin B are added to the 25   substrate most specific for Cathepsin D. Conversely, adding a Cathepsin D inhibitor to an assay for Cathepsin D requires measurement before and after inhibitor addition thus requiring two (2) measurements per assay. The opposite approach only requires one measurement. 30   See Figs. 3A-3D.

Example 20:

Immune Competence

The cell's ability to fight off an invader lies
within its genetics and therefore cell type. The
5 "readiness" however of any genetically capable group of
cells to defend is different.   A measure of this
"readiness" is manifest in the available proteolytic
enzymes contained within vacuoles or on the surface of
the cell. The assay compound hydrolysis rate increases
10 with increased mass of enzymes giving a picture of
immune competence both in number of cells and activity
level. Fig. 4A shows cell size (fs), granularity (ss)
and amino peptidase activity (log fluorescence at 525 nm
v. time) using Leu rhodamine 110-TFA as a substrate in
15 normal Ficoll prepared lymphocytes. Fig. 4B shows the
same data for acute lympholytic Ficoll prepared
lymphocytes. The cells tested in Fig. 4B have lost
their enzymatic activity. Images were generated using
Universal Imaging.

Example 21:

Leukemia

A panel of assay compounds are assembled consisting
of pro-aminopeptidase; aminopeptidase M (Pro, Lys, Gly,
Ala, Leu), Cathepsin D (Gly-Leu, Thr-Pro), Cathepsin B
25 (Gln-Ser, Leu-Gly, Val-Ser, Val-Lys), Cathepsin C (Ala-
Gly) and dipeptidyl peptidase II (Lys-Ala, Gly-Pro, Ala-
Ala). Values for these assay compounds outside the
normal range are considered diagnostic for leukemia. In
addition, the ratios of these enzyme readings to one
30 another provide information on further classifying the
leukemia into myelogenous or lymphocytic and monitoring
the course of the disease. Values may be both higher or
lower than the normal range. Fig. 5A shows results
obtained when normal leukocytes are tested with various
35 rhodamine 110-monopeptide and rhodamine 110-dipeptide
compounds. All compounds except (Lys-Ala)$_2$ rhodamine

110 are TFA salts. (Lys-Ala)$_2$ rhodamine 110 was a free amine derivative. Fig. 5B shows results obtained when leukemia cells are tested with various rhodamine 110-monopeptide compounds.

5

Example 22:

Sepsis

A panel of assay compounds are assembled consisting of aminopeptidase (Leu, Pro, Lys, Gly, Ala), dipeptidyl peptidase II (Gly-Pro, Lys-ala, Ala-Ala), Cathepsin C (Ala-Gly) and Cathepsin B (Leu-Gly, Val-Lys, Val-Ser and Gln-Ser) and cathepsin D (Gly-Leu and Thr-Pro). Values for these substrates outside the normal range are considered diagnostic for sepsis. Fig. 6A shows results obtained when cells from a patient that had been shot by a gun and who was experiencing sepsis were treated with various rhodamine 110-monopeptide and dipeptide compounds. Fig. 6B shows results obtained when umbilical cord blood cells from a newborn infant were treated with various rhodamine 110-monopeptide and dipeptide compounds. All compounds except (Lys-Ala)$_2$ rhodamine 110 are TFA salts. (Lys-Ala)$_2$ rhodamine 110 was a free amine derivative.

Example 23:

TB Infection

A panel of assay compounds are assembled consisting of Ala-aminopeptidase and Lys-aminopeptidase, Dipeptidyl peptidase IV (Ala-Ala)$_2$ rhodamine 110 and Cathepsin D to indicate possible TB infection in AIDS related cases.

A panel of enzymatic substrates is performed consisting of Ala-aminopeptidase and Lys-aminopeptidase, Dipeptidyl peptidase IV (Ala-Ala)$_2$ rhodamine 110 and Cathepsin D to indicate possible TB infection in AIDS related cases. The results are reported in Table 4 below:

TABLE 4

| SUBSTRATE | NORMALS | | | HIV + PATIENTS | | | |
	MEAN DELTA FL	SD	N	MEAN DELTA FL	SD	N	P 2 TAIL
GLN-SER****	11.9	4.4	14	13.7	7.2	13	N.S.
GLN-SER*	6.1	2.6	14	12.7	12.9	17	<0.060
VAL-SER****	24.6	5.3	14	32.5	14.7	7	N.S.
LYS-ALA¹	112.6	43.5	12	121.5	62.9	7	N.S.
LYS-ALA²	7.6	1.7	12	7.9	3.7	13	N.S.
THR-PRO****	295.0	140.9	7	204.4	106.1	6	N.S.
ALA-GLY****	48.0	44.1	11	39.9	43.7	6	N.S.
ALA-GLY*	31.6	27.0	13	29.7	24.8	13	N.S.
THR-PRO****	87.2	95.8	13	62.9	99.2	13	N.S.
THR-PRO*	17.3	18.5	13	10.2	8.7	17	<0.020
GLY-PRO****	46.7	42.8	17	18.4	9.2	13	<0.025
GLY-PRO*	50.9	45.9	17	21.4	15.5	19	<0.020
ALA-ALA****	158.4	47.5	9	110.1	31.6	16	N.S.
ALA-ALA*	25.6	7.6	13	26.3	10.4	13	<0.030
GLY-LEU****	9.5	4.7	17	16.3	11.1	17	N.S.
LEU-GLY**	143.0	132.7	13	104.1	98.4	13	N.S.
LEU-GLY***	162.4	146.9	13	111.9	99.8	13	N.S.
VAL-LYS****	22.7	16.1	15	17.7	10.4	13	N.S.
GLYCINE****	101.1	101.2	11	62.4	73.3	13	<0.095
ALANINE****	132.0	140.4	13	59.0	31.3	17	<0.040
LYSINE****	10.5	3.7	12	7.4	3.8	17	N.S.
PROLINE****	11.4	4.3	10	11.0	5.0	13	<0.020
LEUCINE****	259.6	99.6	8	130.5	99.5	9	<0.020

*   BESTATIN + TFA RHODAMINE 110
**   TARTRATE
***   ACETATE
****   TFA-RHODAMINE 110
1   Rho 110-free amine
2   Rho 110-free amine + bestatin

Example 24:

<u>Metastatic Potential in Solid Tumors</u>

A panel of assay compounds are assembled consisting of Cathepsin B markers (Gln-Ser, Val-Ser), Cathepsin C

5 (Thr-Pro), Dipeptidyl peptidase IV (Ala-Ala) and Leu-aminopeptidase to predict metastatic potential in solid tumors. The results obtained when breast tumor cells (and one normal breast control cell sample) are treated with TFA salts of various rhodamine 110-peptide

10 compounds are shown in Fig. 7.

Example 25:

<u>Monitoring Drug Treatment</u>

An assay compound can be used to monitor drug treatment. Enzymatic activity according to drug target,

15 i.e., protein synthesis, can diminish over time and increase dramatically depending on dose of drug. The results obtained when Raji cells which had been exposed to various concentrations of cyclophosphamide for 48 hours are treated with Leu-rhodamine 110 for 1 minute

20 are shown in Fig. 8A. The results obtained when Raji cells, which had been exposed to various concentrations of vincristine for 48 hours, are treated with a TFA salt of Leu-rhodamine 110 for 1 minute and aminopeptidase activity is measured are shown in Fig. 8B.

25 **Example 26:**

<u>Macrophage Activation</u>

Fig. 9 illustrates the use of assays to provide an indication of macrophage activation. Using a mouse model, various types of cells used to study metastatic

30 versus non-metastatic breast tumors, were treated with Leu-rhodamine 110 substrate and amino peptidase activity was measured. The results obtained are shown in Fig. 9.

Example 27:

Red Blood Cell Ad nosine Deaminase (ADA) and Relationship To Hereditary Non-Spherocytic Hemolytic Anemia (HNSHA) Disease

5   Hereditary deficiencies of glycolytic enzymes or related pathways in the erythrocyte are characterized by the disease hemolytic anemia. Hereditary Non-Spherocytic Hemolytic Anemia is distinguished from Hereditary Spherocytosis by the fact that red blood

10   cells are morphologically normal and manifest a normal osmotic fragility. Only in the case of pyrimidine 5' nucleotidase deficiency is the erythrocyte morphology changed to a basophilic stippling.

Deficiencies of ADA are well-known causes of

15   immunodeficiency. In cases where ADA is greatly increased to levels as high as 100 times normal but other tissues have normal levels in the same individual, the clinical disease is HNSHA. The high ADA depletes the erythrocytes of vital adenine nucleotides, impairing

20   their metabolism. The residual enzyme structure is normal and the gene is normal but attaching the promoter to a reporter gene produced increased levels of enzyme.

The structure of Adenosine is:
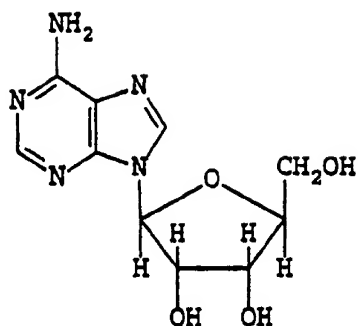
The enzyme adenosine deaminase removes the NH$_2$ and replaces it with a hydroxyl:
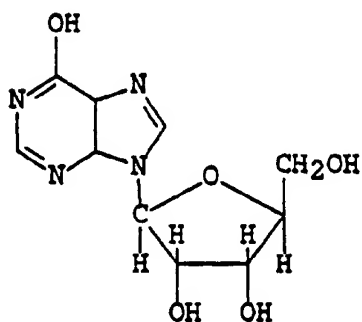
The assay compound using rhodamine 110 is then:
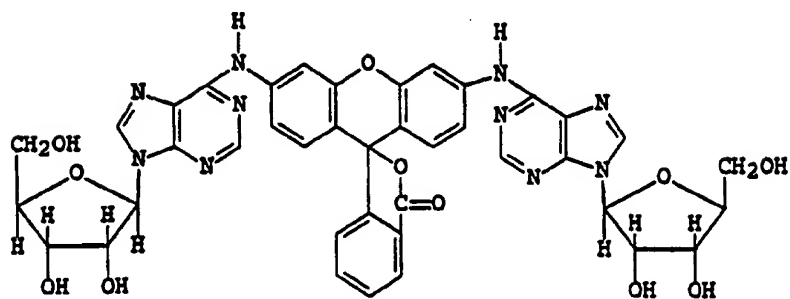
Hydrolysis by ADA leaves 2 Inosine and 1 rhodamine
110:
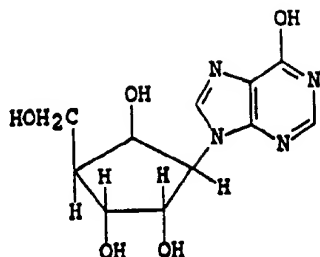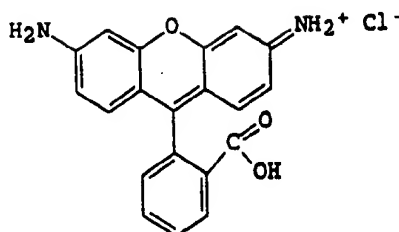
Inosine   Rhodamine 110

To assay vital cells for ADA activity, a blood

5   sample (containing platelets, erythrocytes and leukocytes) is washed to remove plasma, debris, dead cells and extra cellular enzymes. The sample is incubated at 37°C in the wash media.

A media is prepared for the assay reagent using

10   Hanks balanced salts at pH 7.0. The aqueous buffer media is adjusted to isosmotic conditions. The ionic strength is adjusted to 0.1 to 0.3μ by additions of salts. Appropriate cofactors including divalent cations such as Ca^{2+}, Mg^{2+} and Ba^{2+} are added for ADA to maximize

15   the hydrolysis rate. The assay compound is added at excess for the quantities of enzyme analyzed. (A time-course activity assay is used to determine correct fluorescence intensity data collection, usually between 10 seconds and 10 minutes, as well as, appropriate assay

20   compound concentration.)

The washed, pre-incubated blood sample is added to the media, incubated at 37°C and fluorescent intensity is measured at the predetermined time on erythrocytes. The fluorescence found on platelets and leukocytes are

25   disregarded. Separation of cell types is aided by size discrimination.

Comparison of "normal" erythrocytic ADA activity to those in HNSHA disease state demonstrates a 100-fold increase in ADA activity.

The structure of pyrimidine 5'nucleotide is:

5
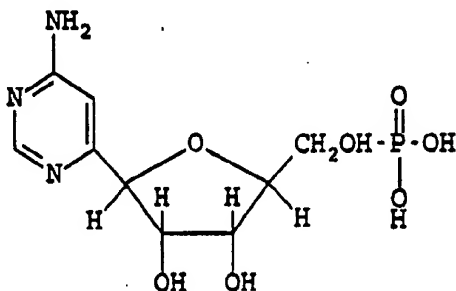
The enzyme pyrimidine 5'nucleotidase removes the phosphate group from the compound:
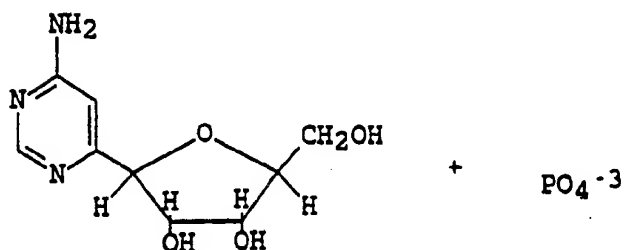
The assay reagent using fluorescein is then:

123
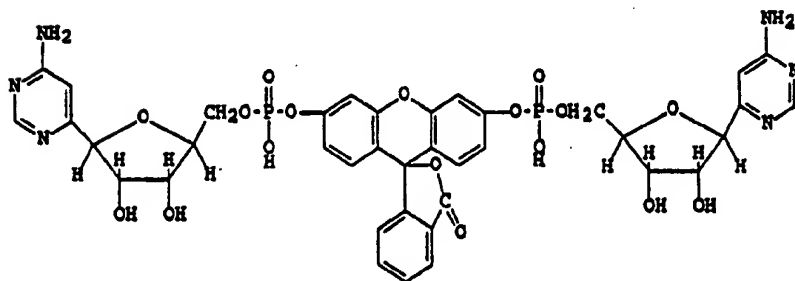
To assay vital cells for pyrimidine-5'-nucleotidase, a blood sample containing platelets, erythrocytes, leukocytes and plasma is washed to remove plasma, debris, dead cells and extracellular enzymes.
5   The sample is pre-incubated at 37°C in the wash media.

A media is prepared for the assay reagent using glycine-sodium hydroxide buffer at pH 8.5. The aqueous buffer media is adjusted to isosmotic conditions. The ionic strength is adjusted to 0.1 to 0.3μ by addition of
10   salts. Appropriate cofactors of calcium chloride and magnesium sulfate are added for pyrimidine 5'nucleotidase to maximize the hydrolysis rate. The assay compound is added at excess for the quantities of enzyme to be analyzed. Other Michaelis-Menten
15   parameters are determined to provide correct data collection window.

The washed pre-incubated blood sample is added to the media, incubated at 37°C and fluorescent intensity is measured on erythrocytes. Erythrocytes are
20   identified visually under a microscope using morphological indicators. Erythrocytes are identified using a flow cytometer by size and granularity discrimination or 2-color assay monoclonal antibody for erythrocytes.
25   Comparison of "normal" erythrocytes pyrimidine 5'nucleotidase to those in HNSHA shows a deficiency of enzyme in HNSHA.

Example 28:
Proinsulin or Pre-proinsulin Inside Cell
30   Insulin is synthesized as a single chain polypeptide - pre-proinsulin. The signal sequence "pre" becomes cleaved during synthesis on the rough endoplasmic reticulum, and no mutations are known that cause disturbances of removing signal sequences, because
35   such mutations probably would be lethal.

Proinsulin is characterized by the presence of a C-peptide that joins the two A and B chains of the mature insulin molecule. Mutations occur mostly at the two critical junctions where the C-peptide is attached to the A and B insulin chains by two pairs of basic amino acids. Such defects have been recognized in families with hyperinsulinemia. The defect is inherited in an autosomal dominant pattern, and probably involves the loss of one of the basic amino acid residues that makes it impossible to cleave the proinsulin molecule at the mutation site, which results in the presence of a two-chained intermediate of proinsulin molecules secreted into the blood plasma.

Clinically occurring glucose intolerance with abnormally high ratios of proinsulin-like material to insulin (9 to 10 as compared with normal values of approximately 0.25) are due to a loss of Arg 65 and loss of the protective activity causing acetylation of Lys 64 which make it impossible to cleave the C-peptide from the A chain of insulin. It is supposed that substitution of Arg 65 of the proinsulin molecule results in failure of cellular enzymes to cleave correctly the C-peptide from the A chain of insulin. Use of a specific substrate, designed for this cleavage site, can be diagnostic for the defect, as well as prognostic for genetic treatment.

Example 29:

Hairy Cell Leukemia

Acid-tartaric buffer in phosphate substrate is used to confirm hairy cell leukemia. "Tartaric Resistant Acid Phosphatase" is a cellular component found in hairy cell leukemia. Design of a buffer system, using tartrate in the buffer and a phosphate substrate, confirms presence of this disease with a positive result.

Example 30:

Cathepsin B

Cathepsin B substrates Gln-Ser, Val-Ser, Leu-Gly, Val-Lys specific for isoenzymes of Cathepsin B. The use
5 of isoelectric focusing for Cathepsin B enzyme and nitrocellulose transfer of these isoenzymes, similar to Western blot, provides a solid support to test substrate activity. Examination of substrate activity, based on design of the dye molecule, determines isoenzyme
10 specificity based on structure.

To prepare the reagent for measuring cathepsin B activity, an assay compound such as Val-Lys-rhodamine 110-TFA is dissolved in 100% DMSO at a stock concentration of 1.6 mM. The stock solution is then
15 diluted 20-fold [with 10 mM MES buffer at pH 6.0] to give a reagent concentration of 0.08 mM. To the 0.08 mM solution the following reagents are added. 0.5 mM Bestatin as an aminopeptidase inhibitor, 1.0 mM dithiothretol as activator, 1.0 mM $CaCl_2$ and 1.0 $MgCl_2$
20 as cofactors and 247 mM Mannitol as a bulking reagent for lyophilization. The complete reagent mixture is lyophilized during which process the DMSO is effectively removed and the lyophilized mixture is reconstituted using endotoxin-free deionized water.

25   **Example 31:**

Response to Modulators

The use of cellular response modifiers, i.e., PMA (phorbol myristate acetate), interleukins and interferons in the pre-incubation step provides
30 information on cellular response. If the cell function is normal the response to the modulator will, for selected enzyme substrates, be in defined ranges. If abnormal, the measured response will be higher or lower.

To make the reagent, the assay compound
35 dichlorofluoroscein diacetate is dissolved in 100% DMSO at 6.0 mM as a stock solution. The stock solution is

diluted 100-fold with 10 mM MES at pH 6.0 to give a reagent concentration of 0.06 mM. 0.032 mM PMA is added as a cell-activator and 247 mM Mannitol is added as a bulking agent for lyophilization. The reagent mixture

5 is lyophilized which effectively removes the DMSO and reconstituted with endotoxin-free deionized water.

Example 32:
Use in Conjunction with Genetic Analysis Techniques

The methods to determine the activity of an enzyme

10 using the assays described above are also useful when used in independent combination with genetic analysis techniques including, polymerase chain reaction (PCR), transcription mediated amplification (TMA), ligase chain reaction (LCR) and fluorescent in situ hybridization

15 (FISH). The results obtained using these genetic analysis techniques can be used both for confirmation of diagnostic conclusions based on measurements of enzymatic activities in cells, as determined with the assays previously described herein, and for the

20 differentiation of purely functional pathologies and functional pathologies having an underlying genetic cause.

Example 33:
Statistical Analysis and Diagnosis of Normal and Diseased States based on Cellular Enzymatic Activity

25

A normal in house adult donor pool was drawn, recording sex and determining that no known disease state was present at the time of sample draw. Criteria

30 for rejecting samples included patients under medication, patients with infection or inflammation of any type, colds and flu as well as any known medical illness such as cancer, heart ailment and high blood pressure. A donor list of 75 patients has been

35 developed and a mean and 2 sd range established. A subgroup of "super normals" was d veloped from this list

by examining the 75 patients to determine which group
falls closest to the mean for all enzymes in all cell
types tested. These patients were used as the "wellness
index" or best group of "normalcy" patients. If a study
5   required the use of children or newborns then this same
format was used to develop a data base for these age
groups. It was noted that the normal range was
different in the different age groups. These groups were
also screened for criteria, for example in the newborns
10   an apgarde value greater than 8, with a normal delivery,
48 hour stay in the hospital and no clinical diseases
diagnosed in the mother was considered normal.

The next task was to develop a similar data base
for different disease states. These were clinically
15   diagnosed using conventional technology to identify the
disease. The patient samples were assayed using the
same protocol as the normal samples. The patients were
not transfused and the sample was less than 4 hours old
at analysis. All cell type information was collected
20   with all enzyme assays. Staging of the disease was
noted where available and all drug treatments were also
recorded. Drug pharmokinetics were determined by the
appropriate reference to estimate the drug's possible
influence on cellular function. Separate studies using
25   the drugs on human tissue culture cell lines were also
used to determine their effect on specific cell types
and provide reference. Untreated patient samples were
separated into their own group and compared to drug
treatment protocols. The goal of the studies was early
30   prediction of disease states as well as monitoring of
treatment modalities.

The data from the collected samples was organized
into tables containing rows of cell type and enzyme
concentration. As illustrated in Table 5, the first
35   seven columns contain either individual patients with a
disease or the mean of patients with a disease.
Calculation of the sum of the squares, the covariance or

the correlation of the rows then indicated which enzyme and what cell type gave the largest difference and therefore the most descriptive indicator of the disease. This approach used the Non-Negative Least Squares method and the ANOVA method. Eigenvectors were then determined for an unknown to predict the most likely disease for the unknown. Fig. 16 shows the prediction of an unknown (actually diagnosed as JRA) from a group of inflammatory diseases (Kawasaki #1, Lupus #2, Juvenile Rheumatoid Arthritis #3, Dermatomyositis #4, Rheumatic Fever #5, and Inflammatory Bowel Disease #6) using the full data matrix versus reduced data matrices using respectively: the enzyme/cell type combinations identified by eigenvector 1 alone, eigenvector 1 and 2 together, or the analysis based on squared deviations from the mean (variance) alone.

TABLE 5

LYMPHOCYTES

SUBSTRATE	Kawasaki's Ratio Dis. Lymphs	Lupus Ratio Lymphs	JRA Ratio Lymphs	Dermatomyositis Ratio Lymphs	Rheumatic Fever Lymphs	Inflam. Bowel Dis. Lymphs	Eigen Vector 0	Eigen Vector 1	Variance	Inflam Dis Lymphs
LEU	0.93	1.93	1.64	1.36	1.05	1.34				0.72
ALA	1.05	1.16	1.59	1.16	1.18	1.53				1.07
GLY	1.94	1.64	0.17	1.30	2.09	1.97				0.16
VK	1.19	1.25	1.60	1.39	0.74	1.08				1.29
VK-M	0.83	0.99	7.71	0.97	0.68	0.66		3	5	4.64
KA	1.53	1.93	0.10	1.39	1.19	1.75				0.07
KA-M	1.32	1.06	1.46	1.93	2.07	1.63				2.06
Z-GP	2.90	1.93	1.29	1.73	3.23	1.73				1.07
Z-GP-M	3.44	2.09	1.30	3.45	4.09	3.80				1.04
Z-TP6.5	5.85	2.30	2.89	3.87	7.19	3.03				1.71
FDA	0.28	1.01	1.17	1.06	0.90	0.87				0.80
FDA-NAF	0.30	0.74	0.21	1.14	0.97	0.89				0.16
DCHFMESPMA	0.09	0.63	0.38	0.52	0.34	0.40				0.31
GFGA	2.15	1.25	2.03	2.14	2.14	2.16				1.40
RGES	1.43	1.84	1.43	2.06	2.04	1.96				1.23
DGLUC	0.25	0.78	0.82	0.72	0.97	1.04				0.45
DPO4	0.62	0.90	0.82	0.64	0.77	0.87				0.45
GALAC	2.46	1.30	2.28	2.34	2.24	2.39				1.40

TABLE 5 (continued)

MONOCYTES

SUBSTRATE	Kawasaki's Dis. Monos	Lupus Ratio Monos	JRA Ratio Monos	Dermatomyositis Ratio Monos	Rheumatic Fever Monos	Inflam. Bowel Dis. Lymphs	Eigen Vector 0	Eigen Vector 1	Variance	Inflam. Dis. Monos
LEU	0.57	44.44	1.44	1.35	0.96	1.13	1		1	0.65
ALA	1.36	6.17	1.31	0.99	1.08	1.43	5			0.94
GLY	3.83	6.00	1.11	1.01	2.06	1.84	6			0.35
VK	5.38	1.64	5.94	1.42	1.32	3.08		7		2.32
VK-M	0.25	11.37	3.49	3.45	8.67	1.41	2	6	3	1.87
KA	2.22	6.93	0.54	1.04	2.39	1.95	4			0.25
KA-M	7.08	14.08	12.14	1.55	15.36	4.10	3	1	2	1.44
Z-GP	4.06	1.88	1.34	1.50	3.56	1.64				0.98
Z-GP-M	4.47	1.92	1.79	3.05	2.99	3.33				0.73
Z-TP6.5	6.40	2.29	8.33	3.12	5.37	2.83		4		3.06
FDA	0.41	1.01	1.55	0.94	0.63	0.98				0.47
FDA-NAF	0.58	0.83	0.35	1.03	0.89	1.11				0.13
DCHFMESPMA	0.13	0.48	0.38	0.47	0.35	0.46				0.24
GFGA	1.77	2.18	2.29	2.01	1.36	3.42				1.67
RGES	4.65	3.03	3.98	5.22	6.52	6.02				0.61
DGLUC	0.35	0.38	1.13	0.73	2.91	4.62				0.28
DPO4	0.51	0.69	1.13	0.79	1.09	1.30				0.28
GALAC	8.40	1.17	3.81	2.14	1.80	4.93			6	1.87

TABLE 5 (continued)

GRANULOCYTES

SUBSTRATE	Kawasaki's Dis. Grans	Lupus Ratio Grans	JRA Ratio Grans	Dermatomyositis Ratio Grans	Rheumatic Fever Grans	Inflam. Bowel Dis. Lymphs	Eigen Vector 0	Eigen Vector 1	Variance	Inflam. Dis. Grans
LEU	0.33	2.03	1.43	1.31	0.98	1.11				0.58
ALA	1.31	1.08	1.21	0.97	1.03	1.26				0.80
GLY	2.30	1.42	0.30	1.12	0.56	1.78				0.17
VK	1.30	1.04	1.26	1.12	0.60	1.08				0.85
VK-M	0.72	0.89	9.49	0.15	0.48	0.64		2	4	3.39
KA	0.99	1.64	0.10	1.16	1.02	1.32				0.05
KA-M	1.32	1.01	1.13	1.02	0.17	1.58				1.37
Z-GP	2.54	1.82	1.24	1.44	2.51	1.47				0.83
Z-GP-M	2.83	1.94	1.46	2.76	2.98	2.68				0.66
Z-TP6.5	4.21	2.19	5.70	2.79	4.73	2.20		5		2.01
FDA	0.31	1.01	1.12	0.92	0.67	0.93				0.50
FDA-NAF	0.39	0.72	0.23	0.97	0.80	0.96				0.08
DCHFMESPMA	0.05	0.31	0.38	0.34	0.14	0.28				0.13
GFGA	1.93	0.10	2.26	1.85	2.10	2.78				1.77
RGES	0.36	1.36	1.01	1.62	1.54	1.60				0.76
DGLUC	0.22	0.77	0.98	1.18	0.13	1.64				0.38
DPO4	0.69	0.71	0.98	0.88	0.24	1.22				0.38
GALAC	2.32	1.01	3.42	1.76	1.16	2.35				1.37

In the above Table the following abbreviations are used:

LEU - (Leu)$_2$ Rho 110

ALA - (Ala)$_2$ Rho 110

5   GLY - (Gly)$_2$ Rho 110

VK - (Val-Lys)$_2$ Rho 110

VK-M - (Val-Lys)$_2$ Rho 110 (modified)

KA - (Lys-Ala)$_2$ Rho 110

KA-M - (Lys-Ala)$_2$ Rho 110

10   Z-GP - (carbobenzyloxycarbonyl-Gly-Pro)$_2$ Rho 110

Z-GP-M - (carbobenzyloxycarbonyl-Gly-Pro)$_2$ Rho 110 (modified)

Z-TP6.5 - (carbobenzyloxycarbonyl-Thr-Pro)$_2$ Rho 110 (pH 6.5)

15   FDA - fluorescein diacetate

FDA-NAF - fluorescein diacetate in a buffer containing sodium fluoride

DCHFMESPMA - dichlorofluorescein in MES buffer plus phorbolmyristate acetate

20   GFGA - (Gly-Phe-Gly-Ala)$_2$ Rho 110

RGES - (Arg-Gly-Glu-Ser)$_2$ Rho 110

DGLUC - (D-glucose)$_2$ fluorescein

DPO4 - (PO$_4$)$_2$ fluorescein

GALAC - (D-galactose)$_2$ fluorescein

25   The reagents designated as "-M" or "modified" contain cofactors, modulators, inhibitors, etc. as shown in Table 1.

As illustrated from Fig. 16, utilizing only the eigenvector 1 and 2 or the squared deviation from the 30 means analysis provides the correct diagnosis, whereas eigenvector 1 alone cannot distinguish from diseases #3 and #4. From Table 5, using three cell types and 18 enzyme concentrations, it is apparent that only six or

eleven values are necessary to classify the unknown sample. The information most informative to the disease diagnosis came from the monocyte cell type and cathepsin, aminopeptidase and dipeptidyl peptidase
5   enzymes.

Example 34:
Analysis and Diagnosis of Normal and Diseased States using Artificial Intelligence

Artificial intelligence was used to analyze data of
10   cellular enzyme functions, and determine normal and disease states.   Peptidases were used to distinguish leukemia from non-leukemia (output pattern). For input patterns lymphocyte and granulocyte cell types were used with aminopeptidases, cathepsins and dipeptidylpeptidase
15   enzyme activities.   In this Example, illustrated in Tables 6A-6C, three normals and three leukemia patients were used as known output patterns for the neural network to learn (in practice, the larger the learning set the more accurate unknown prediction will be).
20   Unknowns were then presented to the learned algorithm as shown in the test case examples. Clinical diagnosis was confirmed by physicians. The trained neural network was able to correctly classify the leukemia from the non-leukemia.

TABLE 6A

THE USE OF PEPTIDASES TO DISTINGUISH
LEUKEMIA FROM NON-LEUKEMIA

Aminopeptidases are Pro, Lys, Gly, Ala; Cathepsin B and Gln/Ser, Val/Ser and
Leu/Gly; Cathepsin C is Thr/.Pro and dipeptidylpeptidase IV is Gly/Pro.

The results of this study show 100% predictability of leukemia when tested
against normal and various diseases as specified.

LEARNED CASES:

Substrates: Pro, Lys, Gly, Ala, Gln/Ser, Thr/Pro, Val/Ser, Leu/Gly and Gly/Pro

Patients:	ID #	DIAGNOSIS**
	1. N191	Normal
	2. N192	Normal
	3. N193	Normal
	4. J4	Acute Leukemia
	5. J5	Acute Myelogenous Leukemia
	6. J4	Acute Leukemia

TEST CASES:

Patients:	ID #	DIAGNOSIS**
	1. J4	Acute Leukemia
	2. J19	Chronic Lymphocytic Leukemia
	3. J22	Acute Myelogenous Leukemia
	4. N199*	Normal
	5. N200	Normal
	6. N201	Normal
	7. N206	Normal
	8. P68	Abnormal - Tachycardia
	9. P69	Abnormal - Pancreatic Cancer
	10. N191	Normal
	11. N192	Normal
	12. N193	Normal
	13. J4	Acute Leukemia
	14. J5	Acute Myelogenous Leukemia
	15. J4	Acute Leukemia
	16. P70	Abnormal - Cirrhosis/Hemobilia
	17. P71	Abnormal - Acute Pyelonephritis
	18. N194	Normal
	19. N195	Normal
	20. N197	Normal
	21. N202	Normal
	22. J22	Acute Myelogenous Leukemia

* Normal donor later found to have cervical cancer
** Diagn sis pr vided by Jackson memorial Hospital; Normal donors were
   in-hous empl yees

TABLE 6B

TEST CASE #4

Synopsis: Lymphs and Grans were used on Learn and Test cases with the following
results:

I.D. #		Negative	Leukemic	Diagnosis
		SCORE		
1	J4		81.4	Acute Leukemia
2	J19		100	Chronic Lymphocytic Leukemia
3	J22		77.9	Acute Myelogenous Leukemia
4	N199*	63.5		Normal
5	N200	100		Normal
6	N201	100		Normal
7	N206	100		Normal
8	P68	100		Tachycardia
9	P69	100		Pancreatic Cancer
10	N191	100		Normal
11	N192	100		Normal
12	N193	100		Normal
13	J4		100	Acute Leukemia
14	J5		99.4	Acute Myelogenous Leukemia
15	J4		99.3	Acute Leukemia
16	P70	100		Cirrhosis/Hemobilia
17	P71	100		Acute Pyelonephritis
18	N194	95.6		Normal
19	N195	100		Normal
20	N197	99		Normal
21	N202	98.5		Normal
22	J22		72.4	Acute Myelogenous Leukemia

PREDICTION: **100%**

	Non-Leukemic	Leukemic
Dx Non-Leukemic	15/15	0/15
Dx Leukemic	0/7	7/7

*Normal donor later found to have cervical cancer

TABLE 6C

CLASSIFICATION OF NEW CASES

J4 11/21/91 ACUTE LEUK.		J5 11/27/91 A M L		P70 CIRRHOSIS/HEMOBILIA	
2.72	GP Lymphs	3.40	GP Lymphs	6.68	GP Lymphs
0.47	PRO L	0.89	PRO L	0.56	PRO L
0.77	LYS L	5.71	LYS L	1.02	LYS L
14.02	GLY L	28.36	GLY L	54.84	GLY L
86.00	ALA L	92.44	ALA L	141.87	ALA L
0.05	QS L	0.07	QS L	0.04	QS L
2.30	TP L	1.32	TP L	1.89	TP L
0.05	VS L	0.05	VS L	0.04	VS L
0.16	LG L	0.23	LG L	0.79	LG L
10.75	GP Grans	8.95	GP Grans	16.42	GP Grans
5.77	PRO G	5.92	PRO G	0.66	PRO G
3.01	LYS G	14.86	LYS G	2.33	LYS G
27.79	GLY G	56.33	GLY G	88.42	GLY G
193.43	ALA G	194.85	ALA G	236.23	ALA G
0.22	QS G	1.24	QS G	0.19	QS G
5.96	TP G	2.72	TP G	4.02	TP G
0.37	VS G	1.13	VS G	0.26	VS G
0.85	LG G	2.51	LG G	2.20	LG G
_0.00_	_NORMAL_	_0.63_	_NORMAL_	_100.00_	_NORMAL_
_100.00_	_LEUKEMIC_	_99.37_	_LEUKEMIC_	_0.00_	_LEUKEMIC_

P71 AC. PYELONEPHRITIS		N194 NORMAL		N195 NORMAL	
3.55	GP Lymphs	5.22	GP Lymphs	5.24	GP Lymphs
0.52	PRO L	0.86	PRO L	0.88	PRO L
0.91	LYS L	0.93	LYS L	1.07	LYS L
41.93	GLY L	39.76	GLY L	48.18	GLY L
109.26	ALA L	97.02	ALA L	118.81	ALA L
0.05	QS L	0.02	QS L	0.04	QS L
1.86	TP L	1.82	TP L	1.90	TP L
0.08	VS L	0.07	VS L	0.06	VS L
0.68	LG L	2.06	LG L	1.93	LG L
11.07	GP Grans	12.49	GP Grans	13.31	GP Grans
6.33	PRO G	3.75	PRO G	4.70	PRO G
4.17	LYS G	1.56	LYS G	2.53	LYS G
59.35	GLY G	60.30	GLY G	79.13	GLY G
185.37	ALA G	164.00	ALA G	221.28	ALA G
0.51	QS G	0.07	QS G	0.21	QS G
4.37	TP G	3.77	TP G	3.74	TP G
0.69	VS G	0.29	VS G	0.37	VS G
4.00	LG G	3.52	LG G	4.11	LG G
_100.00_	_NORMAL_	_95.56_	_NORMAL_	_100.00_	_NORMAL_
_0.00_	_LEUKEMIC_	_4.46_	_LEUKEMIC_	_0.00_	_LEUKEMIC_

TABLE 6C (continued)

N197 NORMAL		N202 NORMAL		J22 12/9/91 A M L	
5.97	GP Lymphs	6.61	GP Lymphs	3.01	GP Lymphs
0.57	PRO L	0.89	PRO L	0.44	PRO L
0.70	LYS L	0.71	LYS L	1.20	LYS L
42.96	GLY L	44.55	GLY L	27.14	GLY L
96.36	ALA L	118.37	ALA L	83.48	ALA L
0.06	QS L	0.01	QS L	0.02	QS L
1.96	TP L	2.49	TP L	1.02	TP L
0.11	VS L	0.04	VS L	0.04	VS L
1.79	LG L	1.28	LG L	0.61	LG L
15.85	GP Grans	14.44	GP Grans	11.52	GP Grans
3.76	PRO G	4.96	PRO G	3.53	PRO G
1.59	LYS G	13.22	LYS G	1.90	LYS G
76.88	GLY G	66.22	GLY G	38.47	GLY G
188.20	ALA G	201.17	ALA G	157.48	ALA G
0.24	QS G	0.08	QS G	0.24	QS G
3.82	TP G	4.44	TP G	2.51	TP G
0.58	VS G	0.24	VS G	0.32	VS G
4.41	LG G	2.70	LG G	1.94	LG G
99.02	*NORMAL*	98.62	*NORMAL*	27.64	*NORMAL*
1.00	*LEUKEMIC*	1.32	*LEUKEMIC*	72.43	*LEUKEMIC*

J4 12/2/91 A L		J4 12/3/91 ACUTE LEUK.		J19 11/27/91 C L L	
3.80	GP Lymphs	4.17	GP Lymphs	1.73	GP Lymphs
0.29	PRO L	0.24	PRO L	0.44	PRO L
1.81	LYS L	1.64	LYS L	0.98	LYS L
27.32	GLY L	31.51	GLY L	11.39	GLY L
68.10	ALA L	74.64	ALA L	47.78	ALA L
0.01	QS L	0.06	QS L	0.06	QS L
1.46	TP L	1.18	TP L	0.50	TP L
0.03	VS L	0.04	VS L	0.01	VS L
0.18	LG L	0.19	LG L	0.02	LG L
11.08	GP Grans	1.28	GP Grans	1.39	GP Grans
3.59	PRO G	3.68	PRO G	5.50	PRO G
4.09	LYS G	3.19	LYS G	4.41	LYS G
47.74	GLY G	48.53	GLY G	48.80	GLY G
139.53	ALA G	138.89	ALA G	143.11	ALA G
0.44	QS G	0.19	QS G	1.16	QS G
2.59	TP G	1.93	TP G	2.95	TP G
0.14	VS G	0.23	VS G	2.83	VS G
0.79	LG G	1.06	LG G	36.35	LG G
0.31	*NORMAL*	18.76	*NORMAL*	0.00	*NORMAL*
99.81	*LEUKEMIC*	81.42	*LEUKEMIC*	100.00	*LEUKEMIC*

TABLE 6C (continued)

J22 12/2/91 A M L		N199* NORMAL		N200 NORMAL	
1.73	GP Lymphs	5.55	GP Lymphs	5.38	GP Lymphs
0.40	PRO L	1.02	PRO L	0.74	PRO L
0.74	LYS L	0.33	LYS L	0.90	LYS L
24.50	GLY L	30.68	GLY L	42.28	GLY L
84.42	ALA L	91.07	ALA L	101.38	ALA L
0.02	QS L	0.02	QS L	0.01	QS L
0.99	TP L	1.86	TP L	2.39	TP L
0.01	VS L	0.03	VS L	0.06	VS L
0.02	LG L	0.85	LG L	1.55	LG L
13.98	GP Grans	13.41	GP Grans	12.74	GP Grans
4.03	PRO G	4.85	PRO G	4.04	PRO G
1.41	LYS G	0.95	LYS G	1.46	LYS G
34.02	GLY G	45.00	GLY G	59.39	GLY G
157.12	ALA G	157.30	ALA G	171.06	ALA G
0.10	QS G	0.05	QS G	0.06	QS G
2.33	TP G	3.71	TP G	4.69	TP G
0.22	VS G	0.14	VS G	0.24	VS G
1.56	LG G	2.02	LG G	3.40	LG G
22.16	*NORMAL*	63.52	*NORMAL*	100.00	*NORMAL*
77.90	*LEUKEMIC*	36.50	*LEUKEMIC*	0.00	*LEUKEMIC*

N201 NORMAL		N206 NORMAL		P68 TACHYCARDIA	
6.53	GP Lymphs	7.09	GP Lymphs	5.92	GP Lymphs
1.10	PRO L	1.28	PRO L	0.70	PRO L
1.05	LYS L	1.65	LYS L	1.51	LYS L
45.32	GLY L	55.56	GLY L	59.29	GLY L
106.72	ALA L	122.71	ALA L	1471.10	ALA L
0.02	QS L	0.04	QS L	0.02	QS L
1.88	TP L	2.89	TP L	1.90	TP L
0.07	VS L	0.28	VS L	0.05	VS L
1.57	LG L	3.12	LG L	0.78	LG L
15.92	GP Grans	17.96	GP Grans	16.08	GP Grans
6.42	PRO G	6.13	PRO G	7.19	PRO G
2.06	LYS G	3.11	LYS G	3.09	LYS G
75.48	GLY G	91.93	GLY G	100.00	GLY G
206.06	ALA G	232.57	ALA G	276.07	ALA G
0.09	QS G	0.27	QS G	0.33	QS G
4.23	TP G	5.98	TP G	4.63	TP G
0.32	VS G	1.03	VS G	0.33	VS G
3.14	LG G	6.01	LG G	2.52	LG G
100.00	*NORMAL*	100.00	*NORMAL*	100.00	*NORMAL*
0.00	*LEUKEMIC*	0.00	*LEUKEMIC*	0.00	*LEUKEMIC*

TABLE 6C (continued)

P69 PANCREATIC CA		N191 NORMAL		N192 NORMAL	
6.80	GP Lymphs	4.14	GP Lymphs	3.00	GP Lymphs
0.66	PRO L	0.69	PRO L	1.14	PRO L
1.22	LYS L	1.18	LYS L	0.56	LYS L
67.38	GLY L	43.57	GLY L	37.47	GLY L
130.34	ALA L	95.72	ALA L	103.31	ALA L
0.31	QS L	0.02	QS L	0.03	QS L
2.26	TP L	2.14	TP L	2.12	TP L
0.18	VS L	0.14	VS L	0.08	VS L
2.00	LG L	2.42	LG L	3.45	LG L
16.21	GP Grans	11.20	GP Grans	11.22	GP Grans
5.70	PRO G	4.34	PRO G	4.34	PRO G
2.14	LYS G	1.20	LYS G	1.20	LYS G
88.04	GLY G	73.20	GLY G	48.67	GLY G
204.02	ALA G	177.32	ALA G	154.84	ALA G
0.37	QS G	0.19	QS G	0.15	QS G
4.21	TP G	4.73	TP G	4.62	TP G
0.57	VS G	0.64	VS G	0.40	VS G
3.53	LG G	5.37	LG G	6.78	LG G
100.00	*NORMAL*	*100.00*	*NORMAL*	*100.00*	*NORMAL*
0.00	*LEUKEMIC*	*0.00*	*LEUKEMIC*	*0.00*	*LEUKEMIC*

N193 NORMAL	
3.00	GP Lymphs
0.95	PRO L
0.52	LYS L
39.30	GLY L
100.50	ALA L
0.03	QS L
2.33	TP L
0.09	VS L
1.56	LG L
8.87	GP Grans
4.75	PRO G
1.43	LYS G
63.33	GLY G
178.64	ALA G
0.14	QS G
4.96	TP G
0.44	VS G
3.55	LG G
98.77	*NORMAL*
1.23	*LEUKEMIC*

TABLE 7

ABERRANT ENZYME ACTIVITIES
IN 10 FEBRILE CHILDREN

LYMPHS

	PATIENTS	P88	P89	P92	P95	P96	P97	P99	P101	P102	P103
	SUBSTRATE										
1	LEU	+	+	+	+	+	o	o	+	o	+
2	ALA	o	o	o			o			o	o
3	PRO			o			o				o
4	LYS			o			o				o
5	GLY	o	−	o			o			o	o
6	SER			o			o				o
7	ARG			o	o	−	+	o		o	o
8	ARG-TFA			o			o				o
9	ASP			o			o				o
10	VS			−	o	o	o	+	−	−	o
11	VS-M			o			o				o
12	VK	o	o	o	−	o	o	+	o	o	o
13	VK-M	o	o	o	+	+	+		o	o	o
14	QS			o			o				o
15	QS-M			o			o				o
16	LG			o	−	−	o	+	−	o	o
17	LG-M			o			o				o
18	KA	o	o	o	o	o	o	o		o	o
19	KA-M	o	o	o	o	o	o	+		o	o
20	Z-AA			−	−	−	−	o	−	o	−
21	Z-AA-M			−			−				−
22	Z-GP	o	−	−	−	−	o		−		−
23	Z-GP-M	−	−	−			−				−
24	GL			o			o				o
25	GL-M			o			o				o
26	Z-AG	−	−	−	−	−	−	o		o	o
27	Z-AG-M			−			−				−
28	AA-TFA			o			o				o
29	AA-M			o			o				o
30	Z-TP6.5	−	−	−			o				o
31	Z-TP6.5M			−			o				o
32	LLR			o			o				o
		Enteritis	Viral Stomatitis	Adenitis	Viral Syndrome	Viral Syndrome	Pyelonephritis	Viral Syndrome	Fever - Unknown	UTI	Viral Syndrome

+   >0.25 Increase over normal range

−   >0.25 Decrease over normal range

o   No Change

TABLE 7 (continued)

LYMPHS

	PATIENTS	P 8 8	P 8 9	P 9 2	P 9 5	P 9 6	P 9 7	P 9 9	P 1 0 1	P 1 0 2	P 1 0 3
	SUBSTRATE										
33	**LLR-M**										
34	**LGLG**			o			o				o
35	**LGLG-M**										
36	**FDA**	o	+	+	+	+	o	o	+	o	+
37	**FDA-NAF**	o	+	+	+	+	o	o	+	o	+
38	**DCFH-DAMES**	o	+	+	+	+	o	o	+	o	+
40	**DCHFMESPMA**	o	+	+	+	+	+	o	+	o	+
42	**GPLGP**	o	o	−			−			o	−
43	**GPLGP-M**			−			o				o
44	**GFGA**	o	o	o			o				o
45	**RGES**	o	o	o	o	+	o	o	o	o	o
46	**DGLUC**	o	o	o							−
47	**DPO4**	o	o	o	o	−	o	o	o	o	o
48	**GALAC**	o	o	o			o				o
49	**TP8.7 M**			−			o				o
50	**TP8.7**			−			o				o
51	**FL GLUC**			o			o				o
		Enteritis	Viral Stomatitis	Adenitis	Viral Syndrome	Viral Syndrome	Pyelonephritis	Viral Syndrome	Fever - Unknown	UTI	Viral Syndrome

+ >0.25 Increase over normal range
− >0.25 Decrease over normal range
o No Change

TABLE 7 (continued)

		MONOS									
	PATIENTS	P88	P89	P92	P95	P96	P97	P99	P101	P102	P103
	SUBSTRATE										
1	LEU	+	+	+	+	+	+	o	+	o	+
2	ALA	o	o	+			+			+	o
3	PRO			o			o				o
4	LYS			+			o				
5	GLY	o	o	o			+				o
6	SER			o			o				+
7	ARG			+	o	o	o	o		o	+
8	ARG-TFA			+			o				+
9	ASP			o			o				o
10	VS			−	−	o	−	+	−	−	o
11	VS-M			−			−				o
12	VK	−	−	o	o	o	o	+	o	−	+
13	VK-M	−	o	−	o	o	o			−	−	o
14	QS			o			o				+
15	QS-M			−			−				o
16	LG			o	−	o	o	+	o		o
17	LG-M			−			o				o
18	KA	o	o	o	o	o	+	o	+	o	o
19	KA-M	−	−	−	−	−	−	o		o	−
20	Z-AA			−	−	o	o	o	−	o	o
21	Z-AA-M			o			o				o
22	Z-GP	o	o	−	o	o	o	o	−	o	o
23	Z-GP-M	−	o	−			o				o
24	GL			o			o				o
25	GL-M			−			o				o
26	Z-AG	−	o	−	o	o	o	o		o	o
27	Z-AG-M			−			o				o
28	AA-TFA						+				+
29	AA-M			−			o				o
30	Z-TP6.5	−	−	−			o				o
		Enteritis	Viral Stomatitis	Adenitis	Viral Syndrome	Viral Syndrome	Pyelonephritis	Viral Syndrome	Fever - Unknown	UTI	Viral Syndrome

+	>0.25 Increase over normal range
−	>0.25 Decrease over normal range
o	No Change

TABLE 7 (continued)

	PATIENTS	MONOS									
		P88	P89	P92	P95	P96	P97	P99	P101	P102	P103
31	Z-TP6.5M			−			o				o
32	LLR			o			o				+
33	LLR-M										
34	LGLG			o			o				o
35	LGLG-M			−			−				+
37	FDA-NAF	o	+	+	+	+	o	+	+		+
38	DCFH-DAMES	o	+	+	+	+	+	o	+	o	+
40	DCHFMESPMA	o	+	+	+	+	+	o	+	o	+
42	GPLGP	−	o	−			−				−
43	GPLGP-M			o			+				+
44	GFGA	o	o	o			o				o
45	RGES	−	o	+	o	o	o	o	−	o	o
46	DGLUC	−	o	−							+
47	DPO4	o	+	+	+		o	o	+	o	+
48	GALAC	o	o	o			o				o
49	TP8.7 M			−			o				o
50	TP8.7			−			o				o
51	FL GLUC			+			o				+
		Enteritis	Viral Stomatitis	Adenitis	Viral Syndrome	Viral Syndrome	Pyelonephritis	Viral Syndrome	Fever - Unknown	UTI	Viral Syndrome
+	>0.25 Increase over normal range										
−	>0.25 Decrease over normal range										
o	No Change										

TABLE 7 (continued)

		ABERRANT ENZYME ACTIVITIES IN 10 FEBRILE CHILDREN									
		GRANS									
	PATIENTS	P88	P89	P92	P95	P96	P97	P99	P101	P102	P103
	SUBSTRATE										
1	LEU	+	+	+	+	+	o	o	+	o	+
2	ALA	o	o	+			+			o	o
3	PRO			o			o				o
4	LYS			+			+				o
5	GLY	o	o	o			o			o	o
6	SER			+			o				o
7	ARG			+	o	o	+	+		o	+
8	ARG-TFA			+			+				+
9	ASP			o			o				o
10	VS			–	–	o	o	+		–	o
11	VS-M			o			o				o
12	VK	–	–	o	–	o	+	+	o	o	o
13	VK-M	–	o	o	+	+	o		o	–	o
14	QS			+			o				+
15	QS-M			+			o				o
16	LG			o	–	o	o	+	o	o	–
17	LG-M			–			–				–
18	KA	o	o	o	o	o	o	o	+	o	o
19	KA-M	o	o	o	o	o	o	o		o	o
20	Z-AA			–	–	o	o	o		o	o
21	Z-AA-M			o			o				o
22	Z-GP	o		–	–	o	o	o			o
23	Z-GP-M	–		–			o				o
24	GL			o			+				o
25	GL-M			o			o				o
26	Z-AG	+	o	o	o	o	o	o		o	o
27	Z-AG-M			–			o				o
28	AA-TFA			+			+				+
29	AA-M			o			o				o
30	Z-TP6.5	–	–	–							o
31	Z-TP6.5M			–			o				o
32	LLR			o			o				o
33	LLR-M										
34	LGLG			o			o				o
		Enteritis	Viral Stomatitis	Adenitis	Viral Syndrome	Viral Syndrome	Pyelonephritis	Viral Syndrome	Fever - Unknown	UTI	Viral Syndrome

+	>0.25 Increase over normal range
–	>0.25 Decrease over normal range
o	No Change

TABLE 7 (continued)

	PATIENTS	GRANS									
		P88	P89	P92	P95	P96	P97	P99	P101	P102	P103
	SUBSTRATE										
35	LGLG-M			o			o				o
36	FDA	o	+	+	+	+	+	o	+	o	+
37	FDA-NAF	o	+	+	+	+	o	+	+	o	+
38	DCFH-DAMES	o	+	+	+	+	+	o	+	o	+
40	DCHFMESPMA	o	+	+	+	+	+	o	+	o	+
42	GPLGP	o	o	−			−			o	−
43	GPLGP-M			−			o				o
44	GFGA	o	o	o			o				o
45	RGES	o	+	+	o	o	o	o	o	o	o
46	DGLUC	o	+	+							+
47	DPO4	o	+	+	o	o	o	o	+	o	+
48	GALAC	o	o	o			o				o
49	TP8.7 M			−			o				o
50	TP8.7			o			o				o
51	FL GLUC			o			o				o
		Enteritis	Viral Stomatitis	Adenitis	Viral Syndrome	Viral Syndrome	Pyelonephritis	Viral Syndrome	Fever - Unknown	UTI	Viral Syndrome

+	>0.25 Increase over normal range
−	>0.25 Decrease over normal range
o	No Change

The use of neural networks has also been used to reduce a large data set (3 cell types x 45 enzyme assays x 5 or more diseases) to only the important cell types and enzyme assays for classification of a disease. This

5   can be visualized by graphing the ratio of the diseases to the mean of the normal of all patients with the disease, as illustrated in Figs. 17A, 17B and 17C to reduce the data set, as illustrated in Table 5, and providing an HLA score sheet, illustrated in Table 7,

10   showing greater than ±25% of the mean normal enzyme activity for that cell type.

Example 35:
Determination of Disease Progression Using
Artificial Intelligence Based Analysis
15   **of Cellular Enzyme Function**

Progression of disease during treatment and monitoring a return to normalcy during treatment are shown in Figs. 18A-18F and the raw data is summarized in Fig. 18G. The time measurements were monitored by

20   three-dimensional plotting of cell-type enzyme activity patterns of the sample values and normal values. Fig. 18A illustrates raw data for lymphocyte cells of a particular patient as compared to the normal data. The raw data in Fig. 18A was taken on August 25. Fig. 18B

25   illustrates the raw data of the diseased patient as compared with normal data on September 6. Similarly, Figs. 18C-18F illustrate raw data for the diseased patient as compared to normal data on September 21, September 26, October 12, and October 13, respectively.

30   The time progression illustrated in Figs. 18A-18F clearly indicates by October 13, the raw data of the "diseased patient" is now virtually identical to the normal data. The increase in stage or complication with additional disease states may also be performed in a

35   manner similar to that illlustrated in Figs. 18A-18F.

All patents and publications referred to in this application are hereby incorporated by reference in their entirety.

The invention has been described with reference to the preferred embodiments. It should be understood, however, that the invention is not so limited, and the scope of the invention should be determined with reference to the following claims, rather than to the foregoing specification.

A first embodiment of the present invention with preferred features can be described by reference to the following numbered paragraphs.

1. An assay reagent for determining the activity of an enzyme in a metabolically active whole cell, said assay reagent comprising at least one water soluble assay compound having the ability to pass through a cell membrane or a water soluble physiologically acceptable salt thereof having the ability to pass through a cell membrane, said assay compound having a leaving group selected for cleavage by an enzyme to be analyzed and a fluorogenic indicator group being selected for its ability to have a non-fluorescent first state when joined to the leaving group, and a fluorescent second state excitable at a wavelength above 450 nm when the leaving group is cleaved from the indicator group by the enzyme, said assay reagent having a fluorescence less than the auto-fluorescence of a metabolically active cell.

2. The assay reagent of Paragraph 1, wherein said leaving group is at least one selected from the group consisting of amino acids, peptides, saccharides, sulfates, phosphates, esters, phosphate esters, nucleotides, polynucleotides, nucleic acids, pyrimidines, purines, nucleosides, lipids and mixtures thereof.

3. The assay reagent of Paragraph 2, wherein said compound in said second state is excitable at a wavelength between 450 and 500 nm and fluoresces at a wavelength of 500-600 nm.

4. The assay reagent of Paragraph 3, wherein said fluorogenic indicator group is selected from the group consisting of rhodamine 110, rhodol, fluorescein, and derivatives thereof which have the 4' or 5' carbon protected.

5. 5. The assay reagent of Paragraph 4, which has a background color of less than 1000 milliabsorbance units.

6. The assay reagent of Paragraph 4, wherein said leaving group is a peptide and the concentration of amino acid and peptide impurities is less than one part per hundred thousand.

7. The assay reagent of Paragraph 4, which has a background fluorescence of less than 100,000 photons.

8. The assay reagent of Paragraph 1, wherein said reagent has an ionic strength between about 0.1 to 0.3 μ.

9. The assay reagent of Paragraph 1, wherein said reagent further includes at least one member selected from the group of a buffer for increasing activity of targeted enzymes relative to non-targeted enzymes, a cofactor for increasing the activity of the enzyme relative to non-targeted enzymes, a modulator for changing the activity of an enzyme, an inhibitor for reducing the activity of non-targeted enzymes, an activator for increasing activity of targeted enzymes over non-targeted enzymes, a solubilizing component, a retention component that inhibits a cell pump mechanism for expressing extracellular material and mixtures thereof.

10. The assay reagent of Paragraph 1, wherein said assay reagent has fluorescence less than the fluorescence generated by about 1×10^{-6} M free indicator groups.

5   11. The assay reagent of Paragraph 1, wherein said salt is an acid salt complex formed from at least one acid selected from the group consisting of hydrochloric, nitric, sulfuric, maleic, acetic, trifluoroacetic, tartaric, citric, succinic, and p-toluenesulfonic acid.

10   A further embodiment of the present invention with preferred features can be described by reference to the following numbered paragraphs.

12. A method to make an assay compound for assaying the activity of an enzyme inside a metabolically active whole cell, said assay compound
15 comprising an indicator group and a leaving group, said leaving group being selected for cleavage by said enzyme, comprising:

        a. reacting a compound containing a leaving group selected from the group consisting of amino acids, peptides, phosphate esters, saccharides, esters, nucleotides, polynucleotides, nucleic acids, pyrimidines, purines,
20 nucleosides, lipids and mixtures thereof and a blocking group, with an agent to form an intermediate complex containing a leaving group and a blocking group;

        b. reacting the intermediate complex with a compound containing an indicator group to form a reaction product;

        c. separating the reaction product from side reaction products, by-
25 products and starting materials;

        d. removing the blocking group from the reaction product to obtain an assay compound having an indicator group and leaving group; and

        e. purifying the assay compound.

13. The method of Paragraph 12, wherein said agent is selected from the group consisting of a substituted carbodiimide, benzotriazolyl-N-oxy-tris(dimethylamino) phosphonium hexafluorophosphate and 1-hydroxybenzo-triazole.

5

14. The method of Paragraph 12, wherein the blocking group is at least one selected from the group consisting of formyl, acetyl, trifluoroacetyl, benzyloxycarbonyl, phthaloyl, benzoyl, acetoacetyl, chloroacetyl, phenoxycarbonyl, carbobenzoxy, substituted benzyloxycarbonyl, 10 tertbutyloxycarbonyl (t-BOC), isopropyloxycarbonyl, allyloxycarbonyl, methoxysuccinyl, succinyl, 2,4-dinitrophenyl, dansyl, p-methoxybenzenesulfonyl, 9-fluorenylmethyloxycarbonyl(FMOC), and phenylthio.

15 15. The method of Paragraph 12, wherein said indicator group is at least one selected from the group consisting of rhodamine 110, rhodol, fluorescein and derivatives thereof which have the 4' or 5' carbon protected.

16. The method of Paragraph 15 wherein the purifying of the assay compound 20 is to a level that background fluorescence of impurities is less than the baseline detection of the enzyme in the cell.

17. The method of Paragraph 15 wherein the purifying of the assay compound is to a level that background fluorescence of the impurities is less than the 25 fluorescence of the metabolically active cell.

18. The method of Paragraph 12 which further comprises reacting the intermediate compound having an indicator group and leaving group with an

acid or a base to form a physiologically acceptable salt of said assay compound for assaying the activity of an enzyme inside a metabolically active whole cell.

19. The method of Paragraph 18, wherein said salt is an acid salt selected from the group consisting of hydrochloric, maleic, acetic, trifluoroacetic, tartaric acid, citric, succinic, and p-toluenesulfonic acid or a base salt selected from the group consisting of ammonia and organic bases.

Another embodiment of the present invention with preferred features can be described by reference to the following numbered paragraphs.

20. A method for determining the activity of an endogenous enzyme in a metabolically active whole cell, comprising:

contacting a metabolically active whole cell with an assay reagent under conditions which allow said assay reagent to pass into said metabolically active whole cell, said assay reagent having at least one assay compound having the ability to pass through a cell membrane or a physiologically acceptable salt thereof having the ability to pass through a cell membrane, said assay compound comprising a fluorogenic indicator group and a leaving group, said leaving group being selected for cleavage by said enzyme, said indicator group being in a non-fluorescent first state when joined to said leaving group, and being in a fluorescent second state excitable at a wavelength above 450 nm when said leaving group is cleaved from said indicator group by said enzyme for a period of time sufficient for said assay reagent to be transferred into said cell and for said leaving group to be cleaved inside said cell from said indicator group by said enzyme;

exposing said cell to light having a wavelength above 450 nm; and

measuring fluorescence of said cell.

21. The m thod of Paragraph 20, wherein said leaving group is at least one selected from the group consisting of amino acids, peptides, saccharides, sulfates, phosphates, esters, phosphate esters, nucleotides, polynucleotides, nucleic acids, pyrimidines, purines, nucleosides, lipids and mixtures thereof.

22. The method of Paragraph 21, wherein said compound in said second state is excitable at a wavelength between 450 and 500 nm and fluoresces at a wavelength of 500-600 nm.

23. The method of Paragraph 22, wherein said fluorogenic indicator group is selected from the group consisting of rhodamine 110, rhodol, fluorescein, and derivatives thereof which have the 4' or 5' carbon protected.

24. The method assay reagent of Paragraph 23, which has a background color of less than 1000 milliabsorbance units.

25. The method assay reagent of Paragraph 23, wherein said leaving group is a peptide and the concentration of amino acid and peptide impurities is less than one part per hundred thousand.

26. The method assay reagent of Paragraph 23, which has a background fluorescence of less than 100,000 photons.

27. The method assay reagent of Paragraph 20, wherein said reagent has an ionic strength between about 0.1 to 0.3 μ.

28. The method assay reagent of Paragraph 20, wherein said reagent further includes at least one member selected from the group of a buffer for increasing activity of targeted enzymes relative to non-targeted enzymes, a cofactor for

increasing the activity of the enzyme relative to non-targeted enzymes, a modulator for changing the activity of an enzyme, an inhibitor for reducing the activity of non-targeted enzymes, an activator for increasing activity of targeted enzymes over non-targeted enzymes, a solubilizing component, a retention

5   component that inhibits a cell pump mechanism for expressing extracellular material and mixtures thereof.

29. The method assay reagent of Paragraph 20, wherein said assay reagent has fluorescence less than the fluorescence generated by about 1×10^{-6} M free

10   indicator groups.

30. The method assay reagent of Paragraph 20, wherein said salt is an acid salt complex formed from at least one acid selected from the group consisting of hydrochloric, nitric, sulfuric, maleic, acetic, trifluoroacetic, tartaric, citric,

15   succinic, and p-toluenesulfonic acid.

31. The method of Paragraph 20, wherein said sensing is performed no more than ten minutes after said cells are contacted with said assay reagent.

20   32. The method of Paragraph 20, wherein said sensing is performed no more than five minutes after said cells are contacted with said assay reagent.

33. The method of Paragraph 20, wherein said reagent is dissolved in a medium, wherein the pH of said medium is between about 4.0 and about 9.5.

25

34. The method of Paragraph 20, wherein said sensing step comprises the measurement of the intensity of said second state against time.

35. The method of Paragraph 20, wherein said sensing step comprises the measurement of the magnitude of said second state at a point of time.

36.. The method of Paragraph 20, wherein said whole cell is washed prior to
5   being contacted with said assay reagent to remove extracellular materials which would interfere with said assay.

An additional embodiment of the present invention with preferred features can be described by reference to the following numbered
10 paragraphs.

37. A method for detecting an abnormality in the activity of an enzyme in a metabolically active whole cell, comprising:

(a)   contacting a reference, metabolically active whole cell having a
15 normally functioning enzyme with a medium containing an assay reagent, said assay reagent having at least one water soluble assay compound having the ability to pass through a cell membrane or a water soluble physiologically acceptable salt thereof having the ability to pass through a cell membrane, said assay compound comprising a fluorogenic indicator group and a leaving group,
20 said leaving group being selected for cleavage by said enzyme, said indicator group being in a non-fluorescent first state when bonded to said leaving group, and being in a fluorescent second state excitable at a wavelength above 450 nm when said leaving group is cleaved from said indicator group by said enzyme, for a period of time sufficient for said assay compound to be
25 transferred into said cell and for said leaving group to be cleaved inside said cell from said indicator group by said enzyme;

(b)   sensing for said fluorescent second state of said indicator group for the reference, metabolically active whole cell to produce reference results;

155

(c)   contacting a test, metabolically active whole cell with said medium for said period of time;

(d)   sensing for said fluorescent second state of said indicator group for the test, metabolically active whole cell to produce test results; and

5   (e)   comparing the reference results of reference test, metabolically active whole cell in said step (b) with the test results obtained from said test metabolically active whole cell in said step (d).

38. The method of paragraph 37, wherein said step (b) includes measuring an

10   intensity of said fluorescent second state against time.

39. The method of paragraph 37, wherein said step (b) includes measuring a magnitude of said fluorescent second state at a point of time.

15   40. The method of paragraph 37, wherein said step (e), the test results and the reference results are compared to obtain a genetic analysis of the test, metabolically active whole cell.

41. The method of paragraph 37, wherein said step (e) is performed by a

20   computer.

42. The method of paragraph 37, said step (e) including performing a Non-Negative Least Squares technique on the test results and the reference results to classify the test results.

25

43. The method of paragraph 37, said step (e) including performing an analysis of variance technique on the test results and the reference results to classify the test results.

44. The method of paragraph 43, wherein the analysis of variance technique includes determining a plurality of eigenvectors and eigenvalues.

45. The method of paragraph 37, said step (e) including performing an analysis
5 of variance technique on the test results and the reference results to obtain a reduced set of test results and reference results.

46. The method of paragraph 45, said step (e) further including performing a non-Negative Least Squares technique on the reduced set of test results and
10 reference results to classify the test results.

47. The method of paragraph 45, said step (e) further including inputting the reduced set of test results and reference results to a Neural Network with back propagation to classify the test results.
15

48. The method of paragraph 45, said step (e) further including inputting the reduced set of test results and reference results to an expert system with Look-Up tables to classify the test results.

20 49. The method of paragraph 41, wherein the computer includes a neural network with back propagation for classifying the test results.

50. The method of paragraph 41, wherein the computer includes an expert system with Look-Up tables for classifying the test results.
25

51. The method of paragraph 37, wherein said step (e) compares the test results and the reference results to diagnose the test, metabolically active whole cell.

52. The method of paragraph 37, wherein said step (e) compares the test results and the reference results to monitor enzyme activity representing morphology or cell type of the test, metabolically active whole cell.

5   53. The method of paragraph 37, wherein said step (e) compares the test results and the reference results to monitor enzyme activity of the test, metabolically active whole cell over time.

54. The method of paragraph 37, wherein said step (e) diagnoses the test, 10 metabolically active whole cell for leukemia, wherein the leaving group includes at least one member selected from the group consisting of Pro, Lys, Gly, Ala, Gln-Ser, Thr-Pro, Val-Ser, Leu-Gly, and Gly-Pro and the test, metabolically active whole cell is a lymphocyte cell or a granulocyte cell.

15   Another further embodiment of the present invention with preferred features can be described by reference to the following numbered paragraphs.

55. A method of performing an assay for detecting the presence of a disease 20 comprising:

(a)   contacting a test, metabolically active whole cell with an assay reagent, said assay reagent containing at least one water soluble assay compound or water soluble physiologically acceptable salt thereof having a fluorogenic indicator group and a leaving group, said leaving group being 25 selected for cleavage by a enzyme the activity of which changes with the presence of the disease, said indicator group being in a non-fluorescent first state when bonded to said leaving group, and being in a fluorescent second state excitable at a wavelength above 450 nm when said leaving group is cleaved from said indicator group by said enzyme for a period of time at least

sufficient for said assay compound to be transferred into said cell and for said leaving group to be cleaved inside said cell from said indicator group by said enzyme;

　　　　　(b)　　sensing for said fluorescent second state of the indicator group
5　for the test, metabolically active whole cell to produce test results; and

　　　　　(c)　　comparing the test results of said test metabolically active whole cell with reference results obtained from at least one of a diseased reference cell and a non-diseased reference cell.

10　56.　The method of paragraph 55, wherein said step (b) includes measuring an intensity of said fluorescent second state against time.

57.　The method of paragraph 55, wherein said step (b) includes measuring a magnitude of said fluorescent second state at a point of time.

15

58.　The method of paragraph 55, wherein said step (c), the test results and the reference results are compared to obtain a genetic analysis of the test, metabolically active whole cell.

20　59.　The method of paragraph 55, wherein said step (c) is performed by a computer.

60.　The method of paragraph 55, said step (c) including performing a non-linear least squares technique on the test results and the reference results to
25　classify the test results.

61.　The method of paragraph 55, said step (c) including performing an analysis of variance technique on the test results and the reference results to classify the test results.

62. The method of paragraph 61, wherein the analysis of variance technique includes determining a plurality of eigenvectors and eigenvalues.

5 63. The method of paragraph 55, said step (c) including performing an analysis of variance technique on the test results and the reference results to obtain a reduced set of test results and reference results.

64. The method of paragraph 63, said step (c) further including performing a

10 Non-Negative Least Squares technique on the reduced set of test results and reference results to classify the test results.

65. The method of paragraph 63, said step (c) further including inputting the reduced set of test results and reference results to a Neural Network with back

15 propagation to classify the test results.

66. The method of paragraph 63, said step (c) further including inputting the reduced set of test results and reference results to an expert system with Look-Up tables to classify the test results.

20

67. The method of paragraph 59, wherein the computer includes a neural network with back propagation for classifying the test results.

68. The method of paragraph 59, wherein the computer includes an expert

25 system with look-up tables for classifying the test results.

69. The method of paragraph 55, wherein said step (c) compares the test results and the reference results to diagnose the test, metabolically active whole cell.

70. The method of paragraph 55, wherein said step (c) compares the test results and the reference results to monitor enzyme activity representing morphology or cell type of the test, metabolically active whole cell.

5

71. The method of paragraph 55, wherein said step (c) compares the test results and the reference results to monitor enzyme activity of the test, metabolically active whole cell over time.

10   72. The method of paragraph 55, wherein said step (c) diagnoses the test, metabolically active whole cell for leukemia, wherein the leaving group includes at least one member selected from the group consisting of Pro, Lys, Gly, Ala, Gln-Ser, Thr-Pro, Val-Ser, Leu-Gly, and Gly-Pro and the test, metabolically active whole cells is a lymphocyte cell or a granulocyte cell.

15

Still further, an supplementary embodiment of the present invention with preferred features can be described by reference to the following numbered paragraphs.

20   73. A method for detecting an abnormality in the activity of an enzyme in a metabolically active whole cell, comprising:

(a)   contacting a plurality of reference, metabolically active whole cells, each having at least one normally functioning enzyme with a medium containing an assay reagent, said assay reagent having at least one water

25   soluble assay compound having the ability to pass through a cell membrane or a water soluble physiologically acceptable salt thereof having the ability to pass through a cell membrane, said assay compound comprising a fluorogenic indicator group and a leaving group, said leaving group being selected for cleavage by one of said at least one normally functioning enzymes, said

indicator group being in a non-fluorescent first state when bonded to said leaving group, and being in a fluorescent second state excitable at a wavelength above 450 nm when said leaving group is cleaved from said indicator group by the one of said at least one normally functioning enzyme, for

5   a period of time sufficient for said assay compound to be transferred into each of said plurality of reference, metabolically active whole cells for each of the at least one normally functioning enzymes for each of said plurality of reference, metabolically active whole cells to produce a matrix of reference results and for said leaving group to be cleaved inside of each of said plurality of reference,

10   metabolically active whole cells from said indicator group by the one of said at least one normally functioning enzymes;

(b)   sensing for said fluorescent second state of said indicator group for each of the at least one normally functioning enzymes for each said plurality of reference, metabolically active whole cells to produce a matrix of reference

15   results;

(c)   contacting a plurality of test, metabolically active whole cells, each having at least one normally functioning enzyme with said medium for said period of time;

(d)   sensing for said fluorescent second state of said indicator group

20   for each of the at least one normal functioning enzyme for each of said plurality of test, metabolically active whole cells to produce a matrix of test results; and

(e)   comparing the matrix of test results of said plurality of test, metabolically active whole cells in said step (d) with the matrix of reference results obtained from said plurality of reference, metabolically active whole cells

25   in said step (b).

74. The method of paragraph 73, wherein said step (b) includes measuring an intensity of said fluorescent second state against time.

75. The method of paragraph 73, wherein said step (b) includes measuring a magnitude of said fluorescent second state at a point of time.

76. The method of paragraph 73, wherein said step (e), the matrix of test
5 results and the matrix of reference results are compared to obtain a genetic analysis of the plurality of test, metabolically active whole cells.

77. The method of paragraph 73, wherein said step (e) is performed by a computer.
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78. The method of paragraph 73, said step (e) including performing a Non-Negative Least Squares technique on the matrix of test results and the matrix of reference results to classify the matrix of test results.

15 79. The method of paragraph 73, said step (e) including performing an Analysis of Variance technique on the matrix of test results and the matrix of reference results to classify the matrix of test results.

80. The method of paragraph 79, wherein the analysis of variance techniques
20 includes determining a plurality of eigenvectors and eigenvalues.

81. The method of paragraph 73, said step (e) including performing an analysis of variance technique on the test results and the reference results to obtain a reduced set of test results and reference results.
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82. The method of paragraph 81, said step (e) further including performing a Non-Negative Least Squares technique on the reduced set of test results and reference results to classify the test results.

83. The method of paragraph 81, said step (e) further including inputting the required set of test results and reference results to a Neural Network with back propagation to classify the test results.

5   84. The method of paragraph 81, said step (e), further including inputting the reduced set of test results and reference results to an expert system with Look-Up tables to classify the test results.

85. The method of paragraph 77, wherein the computer includes a Neural
10   Network with back propagation for classifying the matrix of test results.

86. The method of paragraph 77, wherein the computer includes an expert system with Look-Up tables for classifying the matrix of test results.

15   87. The method of paragraph 73, wherein said step (e) compares the matrix of test results and the matrix of reference results to diagnose the plurality of test, metabolically active whole cells.

88. The method of paragraph 73, wherein said step (e) compares the matrix of
20   test results and the matrix of reference results to monitor enzyme activity representing morphology or cell type of the plurality of test, metabolically active whole cells.

89. The method of paragraph 73, wherein said step (e) compares the matrix of
25   test results and the matrix of reference results to monitor enzyme activity of the plurality of test, metabolically active whole cells over time.

90. The method of paragraph 73, wherein said step (e) diagnoses the plurality of test, metabolically active whole cells for leukemia, wherein th   at least on

normally functioning enzymes include Pro, Lys, Gly, Ala, Gln-Ser, Thr-Pro, Val-Ser, Leu-Gly, and Gly-Pro and the plurality of test, metabolically active whole cells include lymphocyte cells and granulocyte cells.

5   An added embodiment of the present invention with preferred features can be described by reference to the following numbered paragraphs.

91. A method of performing an assay for detecting the presence of a disease
10   comprising:

(a)   contacting a test, metabolically active whole cell with an assay reagent, said assay reagent containing at least one water soluble assay compound or water soluble physiologically acceptable salt thereof having a fluorogenic indicator group and a leaving group, said leaving group being
15   selected for cleavage by one of said at least one normally functioning enzyme the activity of which changes with the presence of the disease, said indicator group being in a non-fluorescent first state when bonded to said leaving group, and being in a fluorescent second state excitable at a wavelength above 450 nm when said leaving group is cleaved from said indicator group by the one of
20   said at least one normally functioning enzyme for a period of time at least sufficient for said assay compound to be transferred into said cell and for said leaving group to be cleaved inside said cell from said indicator group by said enzyme;

(b)   sensing for said fluorescent second state of the indicator group
25   for the test, metabolically active whole cell to produce a matrix of test results; and

(c)   comparing the matrix of test results of said test metabolically active whole cell with a matrix of reference results obtained from at least one of a diseased reference cell and a non-diseased reference cell.

92. The method of paragraph 91, wherein said step (b) includes measuring an intensity of said fluorescent second state against time.

5 93. The method of paragraph 91, wherein said step (b) includes measuring a magnitude of said fluorescent second state at a point of time.

94. The method of paragraph 91, wherein in said step (c), the test results and the reference results are compared to obtain a genetic analysis of the test,
10 metabolically active whole cell.

95. The method of paragraph 91, wherein said step (c) is performed by a computer.

15 96. The method of paragraph 91, said step (c) including performing a Non-Negative Least Squares technique on the matrix of test results and the matrix of reference results to classify the matrix of test results.

97. The method of paragraph 91, said step (c) including performing an Analysis
20 of Variance technique on the matrix of test results and the matrix of reference results to classify the matrix of test results.

98. The method of paragraph 97, wherein the analysis of variance technique includes detecting a plurality of eigenvectors and eigenvalues.
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99. The method of paragraph 91, said step (c) including performing an analysis of variance technique on the test results and the reference results to obtain a reduced set of test results and reference results.

100. The method of paragraph 98, said step (c) further including performing a Non-Negative Least Squares technique on the reduced set of test results and reference results to classify the test results.

5   101. The method of paragraph 98, said step (c) further including inputting the reduced set of test results and reference results to a Neural Network with back propagation to classify the test results.

102. The method of paragraph 98, said step (c) further including inputting the

10   reduced set of test results and reference results to an expert system with Look-Up tables to classify the test results.

103. The method of paragraph 95, wherein the computer includes a Neural Network with back propagation for classifying the matrix of test results.

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104. The method of paragraph 105, wherein the computer includes an expert system with Look-Up tables for classifying the matrix of test results.

105. The method of paragraph 91, wherein said step (c) compares the matrix of

20   test results and the matrix of reference results to diagnose the test, metabolically active whole cell.

106. The method of paragraph 91, wherein said step (c) compares the matrix of test results and the matrix of reference results to monitor enzyme activity

25   representing morphology or cell type of the test, metabolically active whole cell.

107. The method of paragraph 91, wherein said step (c) compares the matrix of test results and the matrix of reference results to monitor enzyme activity of the test, metabolically active whole cell over time.

108. The method of paragraph 91, wherein said step (c) diagnoses the test, metabolically active whole cell for leukemia, wherein the at least one normally functioning enzymes include Pro, Lys, Gly, Ala, Gln-Ser, Thr-Pro, Val-Ser, Leu-

5 Gly, and Gly-Pro and the test, metabolically active whole cell is a lymphocyte cell or a granulocyte cell.

109. The method of paragraph 20, wherein cells are classified into cell types after determining the activity of said endogenous enzyme.

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110. The method of paragraph 109, wherein enzymes are measured which distinguish nucleated red blood cells from non-nucleated red blood cells.

111. The method of paragraph 20, wherein cells obtained from a patient are

15 tested for the presence of one or more of said endogenous enzymes to determine whether said patient has a disease or abnormal condition.

112. The method of paragraph 20, wherein said disease or abnormal condition is selected from the group consisting of cancer, infection, sepsis, immune

20 disorder and anemia.

113. The method of paragraph 20, wherein said disease or abnormal condition which is measured or monitored is selected from the group consisting of hairy cell leukemia, chronic lymphocytic leukemia, B-cell leukemia, infection by

25 bacteria, virus, fungus or yeast, sepsis, inflammatory conditions, corticosteroid treatment, AIDS, tuberculosis, metastatic potential of solid tumors, activation and/or modulation of macrophages, hereditary non-spherocytic hemolytic anemia, female reproductive tract cancer, breast tumor, colon cancer, Werthen's disease, pancreatic cancer, ovarian cancer, uterine cancer, cervical

cancer, Gaucher's disease, Fabry disease, Tay Sach's, Wolman's disease, prostatic cancer, adenosine deaminase deficiency and lymphoma.

114. The method of paragraph 20, wherein an infection selected from the group consisting of AIDS, cytomegalovirus (CMV), herpes, hepatitis, and syphilis is monitored.

115. The method of paragraph 20, wherein a cell type selected from the group consisting of mature erythrocytes, nucleated erythrocytes, T-lymphocytes, B-lymphocytes, NK lymphs, atypical lymphs, eosinophils, lymphoid blasts, myeloid blasts, monocytic blasts, promonocytes, large mature monocytes, macrophages in a resting state, activated macrophages, elicited macrophages, basophils, promyelocyte, myelocyte, bands, mature neutrophils, platelets, neutrophil toxic granulation, neutrophil non-toxic granulation, platelet clumps, megakaryocytes, and reactive monocytes is determined.

116. The method of paragraph 76 or 94, which further comprises performing genetic analysis by PCR on test cells.

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We Claim:

1.   An assay reagent for determining the activity of an enzyme in a metabolically active whole cell, said assay reagent comprising at least one water soluble assay compound having the ability to pass through a cell membrane or a water soluble physiologically acceptable salt thereof having the ability to pass through a cell membrane, said assay compound having a leaving group selected for cleavage by an enzyme to be analyzed and a fluorogenic indicator group being selected for its ability to have a non-fluorescent first state when joined to the leaving group, and a fluorescent second state excitable at a wavelength above 450 nm when the leaving group is cleaved from the indicator group by the enzyme, said assay reagent having a fluorescence less than the auto-fluorescence of a metabolically active cell.

2.   The assay reagent of Claim 1, wherein said leaving group is at least one selected from the group consisting of amino acids, peptides, saccharides, sulfates, phosphates, esters, phosphate esters, nucleotides, polynucleotides, nucleic acids, pyrimidines, purines, nucleosides, lipids and mixtures thereof.

3.   The assay reagent of Claim 2, wherein said compound in said second state is excitable at a wavelength between 450 and 500 nm and fluoresces at a wavelength of 500-600 nm.

4.   The assay reagent of Claim 3, wherein said fluorogenic indicator group is selected from the group consisting of rhodamine 110, rhodol, fluorescein, and derivatives thereof which have the 4' or 5' carbon protected.

5.   The assay reagent of Claim 4, which has a background color of less than 1000 milliabsorbance units.

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6. The assay reagent of Claim 4, wherein said leaving group is a peptide and the concentration of amino acid and peptide impurities is less than one part per hundred thousand.

7. The assay reagent of Claim 4, which has a background fluorescence of less than 100,000 photons.

8. The assay reagent of Claim 1, wherein said reagent has an ionic strength between about 0.1 to 0.3 μ.

9. The assay reagent of Claim 1, wherein said reagent further includes at least one member selected from the group of a buffer for increasing activity of targeted enzymes relative to non-targeted enzymes, a cofactor for increasing the activity of the enzyme relative to non-targeted enzymes, a modulator for changing the activity of an enzyme, an inhibitor for reducing the activity of non-targeted enzymes, an activator for increasing activity of targeted enzymes over non-targeted enzymes, a solubilizing component, a retention component that inhibits a cell pump mechanism for expressing extracellular material and mixtures thereof.

10. The assay reagent of Claim 1, wherein said assay reagent has fluorescence less than the fluorescence generated by about 1×10^{-6} M free indicator groups.

11. The assay reagent of Claim 1, wherein said salt is an acid salt complex formed from at least one acid selected from the group consisting of hydrochloric, nitric, sulfuric, maleic, acetic, trifluoroacetic, tartaric, citric, succinic, and p-toluenesulfonic acid.

12.   A method to make an assay compound for assaying the activity of an enzyme inside a metabolically active whole cell, said assay compound comprising an indicator group and a leaving group, said leaving group being selected for cleavage by said enzyme, comprising:

a.   reacting a compound containing a leaving group selected from the group consisting of amino acids, peptides, phosphate esters, saccharides, esters, nucleotides, polynucleotides, nucleic acids, pyrimidines, purines, nucleosides, lipids and mixtures thereof and a blocking group, with an agent to form an intermediate complex containing a leaving group and a blocking group;

b.   reacting the intermediate complex with a compound containing an indicator group to form a reaction product;

c.   separating the reaction product from side reaction products, by-products and starting materials;

d.   removing the blocking group from the reaction product to obtain an assay compound having an indicator group and leaving group; and

e.   purifying the assay compound.

13.   The method of Claim 12, wherein said agent is selected from the group consisting of a substituted carbodiimide, benzotriazolyl-N-oxy-tris(dimethylamino) phosphonium hexafluorophosphate and 1-hydroxybenzo-triazole.

14.   The method of Claim 12, wherein the blocking group is at least one selected from the group consisting of formyl, acetyl, trifluoroacetyl, benzyloxycarbonyl, phthaloyl, benzoyl, acetoacetyl, chloroacetyl, phenoxycarbonyl, carbobenzoxy, substituted benzyloxycarbonyl, tertbutyloxycarbonyl (t-BOC), isopropyloxycarbonyl, allyloxycarbonyl, methoxysuccinyl, succinyl, 2,4-dinitrophenyl, dansyl, p-methoxybenzenesulfonyl, 9-fluorenylm thyloxycarbonyl(FMOC),
and phenylthio.

15.   The method of Claim 12, wherein said indicator group is at least one selected from the group consisting of rhodamine 110, rhodol, fluorescein and derivatives thereof which have the 4' or 5' carbon protected.

16.   The method of Claim 15 wherein the purifying of the assay compound is to a level that background fluorescence of impurities is less than the baseline detection of the enzyme in the cell.

17.   The method of Claim 15 wherein the purifying of the assay compound is to a level that background fluorescence of the impurities is less than the fluorescence of the metabolically active cell.

18.   The method of Claim 12 which further comprises reacting the intermediate compound having an indicator group and leaving group with an acid or a base to form a physiologically acceptable salt of said assay compound for assaying the activity of an enzyme inside a metabolically active whole cell.

19.   The method of Claim 18, wherein said salt is an acid salt selected from the group consisting of hydrochloric, maleic, acetic, trifluoroacetic, tartaric acid, citric, succinic, and p-toluenesulfonic acid or a base salt selected from the group consisting of ammonia and organic bases.

20.   A method for determining the activity of an endogenous enzyme in a metabolically active whole cell, comprising:

        contacting a metabolically active whole cell with an assay reagent under conditions which allow said assay reagent to pass into said metabolically active whole cell, said assay reagent having at least one assay compound having the ability to pass through a cell membrane or a physiologically acceptable salt thereof having the ability to pass through a cell membrane, said assay compound

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comprising a fluorogenic indicator group and a leaving group, said leaving group being selected for cleavage by said enzyme, said indicator group being in a non-fluorescent first state when joined to said leaving group, and being in a fluorescent second state excitable at a wavelength above 450 nm when said leaving group is cleaved from said indicator group by said enzyme for a period of time sufficient for said assay reagent to be transferred into said cell and for said leaving group to be cleaved inside said cell from said indicator group by said enzyme;

exposing said cell to light having a wavelength above 450 nm; and

measuring fluorescence of said cell.

21.   The method of Claim 20, wherein said leaving group is at least one selected from the group consisting of amino acids, peptides, saccharides, sulfates, phosphates, esters, phosphate esters, nucleotides, polynucleotides, nucleic acids, pyrimidines, purines, nucleosides, lipids and mixtures thereof.

22.   The method of Claim 21, wherein said compound in said second state is excitable at a wavelength between 450 and 500 nm and fluoresces at a wavelength of 500-600 nm.

23.   The method of Claim 22, wherein said fluorogenic indicator group is selected from the group consisting of rhodamine 110, rhodol, fluorescein, and derivatives thereof which have the 4' or 5' carbon protected.

24.   The method assay reagent of Claim 23, which has a background color of less than 1000 milliabsorbance units.

25.   The method assay reagent of Claim 23, wherein said leaving group is a peptide and the concentration of amino acid and peptide impurities is less than one part per hundred thousand.

26.   The method assay reagent of Claim 23, which has a background fluorescence of less than 100,000 photons.

27.   The method assay reagent of Claim 20, wherein said reagent has an ionic strength between about 0.1 to 0.3 μ.

28.   The method assay reagent of Claim 20, wherein said reagent further includes at least one member selected from the group of a buffer for increasing activity of targeted enzymes relative to non-targeted enzymes, a cofactor for increasing the activity of the enzyme relative to non-targeted enzymes, a modulator for changing the activity of an enzyme, an inhibitor for reducing the activity of non-targeted enzymes, an activator for increasing activity of targeted enzymes over non-targeted enzymes, a solubilizing component, a retention component that inhibits a cell pump mechanism for expressing extracellular material and mixtures thereof.

29.   The method assay reagent of Claim 20, wherein said assay reagent has fluorescence less than the fluorescence generated by about 1×10^{-6} M free indicator groups.

30.   The method assay reagent of Claim 20, wherein said salt is an acid salt complex formed from at least one acid selected from the group consisting of hydrochloric, nitric, sulfuric, maleic, acetic, trifluoroacetic, tartaric, citric, succinic, and p-toluenesulfonic acid.

31.   The method of Claim 20, wherein said sensing is performed no more than ten minutes after said cells are contacted with said assay reagent.

32.   The method of Claim 20, wherein said sensing is performed no more than five minutes after said cells are contacted with said assay reagent.

33.   The method of Claim 20, wherein said reagent is dissolved in a medium, wherein the pH of said medium is between about 4.0 and about 9.5.

34.   The method of Claim 20, wherein said sensing step comprises the measurement of the intensity of said second state against time.

35.   The method of Claim 20, wherein said sensing step comprises the measurement of the magnitude of said second state at a point of time.

36.   The method of Claim 20, wherein said whole cell is washed prior to being contacted with said assay reagent to remove extracellular materials which would interfere with said assay.

37.   A method for detecting an abnormality in the activity of an enzyme in a metabolically active whole cell, comprising:

(a)   contacting a reference, metabolically active whole cell having a normally functioning enzyme with a medium containing an assay reagent, said assay reagent having at least one water soluble assay compound having the ability to pass through a cell membrane or a water soluble physiologically acceptable salt thereof having the ability to pass through a cell membrane, said assay compound comprising a fluorogenic indicator group and a leaving group, said leaving group being selected for cleavage by said enzyme, said indicator group being in a non-fluorescent first state when bonded to said leaving group, and being in a fluorescent second state excitable at a wavelength above 450 nm when said leaving group is cleaved from said indicator group by said enzyme, for a period of time sufficient for said assay compound to b   transferred into said cell

and for said leaving group to be cleaved inside said cell from said indicator group by said enzyme;

(b)   sensing for said fluorescent second state of said indicator group for the reference, metabolically active whole cell to produce reference results;

(c)   contacting a test, metabolically active whole cell with said medium for said period of time;

(d)   sensing for said fluorescent second state of said indicator group for the test, metabolically active whole cell to produce test results; and

(e)   comparing the reference results of reference test, metabolically active whole cell in said step (b) with the test results obtained from said test metabolically active whole cell in said step (d).

38.   The method of Claim 37, wherein said step (b) includes measuring an intensity of said fluorescent second state against time.

39.   The method of Claim 37, wherein said step (b) includes measuring a magnitude of said fluorescent second state at a point of time.

40.   The method of Claim 37, wherein said step (e), the test results and the reference results are compared to obtain a genetic analysis of the test, metabolically active whole cell.

41.   The method of Claim 37, wherein said step (e) is performed by a computer.

42.   The method of Claim 37, said step (e) including performing a Non-Negative Least Squares technique on the test results and the reference results to classify the test results.

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43.   The method of Claim 37, said step (e) including performing an analysis of variance technique on the test results and the reference results to classify the test results.

44.   The method of Claim 43, wherein the analysis of variance technique includes determining a plurality of eigenvectors and eigenvalues.

45.   The method of Claim 37, said step (e) including performing an analysis of variance technique on the test results and the reference results to obtain a reduced set of test results and reference results.

46.   The method of Claim 45, said step (e) further including performing a Non-Negative Least Squares technique on the reduced set of test results and reference results to classify the test results.

47.   The method of Claim 45, said step (e) further including inputting the reduced set of test results and reference results to a neural network with back propagation to classify the test results.

48.   The method of Claim 45, said step (e) further including inputting the reduced set of test results and reference results to an expert system with Look-Up tables to classify the test results.

49.   The method of Claim 41, wherein the computer includes a neural network with back propagation for classifying the test results.

50.   The method of Claim 41, wherein the computer includes an expert system with Look-Up tables for classifying the test results.

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51.   The method of Claim 37, wherein said step (e) compares the test results and the reference results to diagnose the test, metabolically active whole cell.

52.   The method of Claim 37, wherein said step (e) compares the test results and the reference results to monitor enzyme activity representing morphology or cell type of the test, metabolically active whole cell.

53.   The method of Claim 37, wherein said step (e) compares the test results and the reference results to monitor enzyme activity of the test, metabolically active whole cell over time.

54.   The method of Claim 37, wherein said step (e) diagnoses the test, metabolically active whole cell for leukemia, wherein the leaving group includes at least one member selected from the group consisting of Pro, Lys, Gly, Ala, Gln-Ser, Thr-Pro, Val-Ser, Leu-Gly, and Gly-Pro and the test, metabolically active whole cell is a lymphocyte cell or a granulocyte cell.

55.   A method of performing an assay for detecting the presence of a disease comprising:

(a)   contacting a test, metabolically active whole cell with an assay reagent, said assay reagent containing at least one water soluble assay compound or water soluble physiologically acceptable salt thereof having a fluorogenic indicator group and a leaving group, said leaving group being selected for cleavage by a enzyme the activity of which changes with the presence of the disease, said indicator group being in a non-fluorescent first state when bonded to said leaving group, and being in a fluorescent second state excitable at a wavelength above 450 nm when said leaving group is cleaved from said indicator group by said enzyme for a period of tim   at least sufficient for said

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assay compound to be transferred into said cell and for said leaving group to be cleaved inside said cell from said indicator group by said enzyme;

(b)   sensing for said fluorescent second state of the indicator group for the test, metabolically active whole cell to produce test results; and

(c)   comparing the test results of said test metabolically active whole cell with reference results obtained from at least one of a diseased reference cell and a non-diseased reference cell.

56.   The method of Claim 55, wherein said step (b) includes measuring an intensity of said fluorescent second state against time.

57.   The method of Claim 55, wherein said step (b) includes measuring a magnitude of said fluorescent second state at a point of time.

58.   The method of Claim 55, wherein said step (c), the test results and the reference results are compared to obtain a genetic analysis of the test, metabolically active whole cell.

59.   The method of Claim 55, wherein said step (c) is performed by a computer.

60.   The method of Claim 55, said step (c) including performing a non-linear least squares technique on the test results and the reference results to classify the test results.

61.   The method of Claim 55, said step (c) including performing an analysis of variance technique on the test results and the reference results to classify the test results.

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62. The method of Claim 61, wherein the analysis of variance technique includes determining a plurality of eigenvectors and eigenvalues.

63. The method of Claim 55, said step (c) including performing an analysis of variance technique on the test results and the reference results to obtain a reduced set of test results and reference results.

64. The method of Claim 63, said step (c) further including performing a Non-Negative Least Squares technique on the reduced set of test results and reference results to classify the test results.

65. The method of Claim 63, said step (c) further including inputting the reduced set of test results and reference results to a Neural Network with back propagation to classify the test results.

66. The method of Claim 63, said step (c) further including inputting the reduced set of test results and reference results to an expert system with Look-Up tables to classify the test results.

67. The method of Claim 59, wherein the computer includes a neural network with back propagation for classifying the test results.

68. The method of Claim 59, wherein the computer includes an expert system with look-up tables for classifying the test results.

69. The method of Claim 55, wherein said step (c) compares the test results and the reference results to diagnose the test, metabolically active whole cell.

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70.   The method of Claim 55, wherein said step (c) compares the test results and the reference results to monitor enzyme activity representing morphology or cell type of the test, metabolically active whole cell.

71.   The method of Claim 55, wherein said step (c) compares the test results and the reference results to monitor enzyme activity of the test, metabolically active whole cell over time.

72.   The method of Claim 55, wherein said step (c) diagnoses the test, metabolically active whole cell for leukemia, wherein the leaving group includes at least one member selected from the group consisting of Pro, Lys, Gly, Ala, Gln-Ser, Thr-Pro, Val-Ser, Leu-Gly, and Gly-Pro and the test, metabolically active whole cells is a lymphocyte cell or a granulocyte cell.

73.   A method for detecting an abnormality in the activity of an enzyme in a metabolically active whole cell, comprising:

(a)   contacting a plurality of reference, metabolically active whole cells, each having at least one normally functioning enzyme with a medium containing an assay reagent, said assay reagent having at least one water soluble assay compound having the ability to pass through a cell membrane or a water soluble physiologically acceptable salt thereof having the ability to pass through a cell membrane, said assay compound comprising a fluorogenic indicator group and a leaving group, said leaving group being selected for cleavage by one of said at least one normally functioning enzymes, said indicator group being in a non-fluorescent first state when bonded to said leaving group, and being in a fluorescent second state excitable at a wavelength above 450 nm when said leaving group is cleaved from said indicator group by the one of said at least one normally functioning enzyme, for a period of time sufficient for said assay compound to be transferred into each of said plurality of reference, metabolically active whole cells for each of the at least one normally functioning enzym s for

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each of said plurality of reference, metabolically active whole cells to produce a matrix of reference results and for said leaving group to be cleaved inside of each of said plurality of reference, metabolically active whole cells from said indicator group by the one of said at least one normally functioning enzymes;

(b)   sensing for said fluorescent second state of said indicator group for each of the at least one normally functioning enzymes for each said plurality of reference, metabolically active whole cells to produce a matrix of reference results;

(c)   contacting a plurality of test, metabolically active whole cells, each having at least one normally functioning enzyme with said medium for said period of time;

(d)   sensing for said fluorescent second state of said indicator group for each of the at least one normal functioning enzyme for each of said plurality of test, metabolically active whole cells to produce a matrix of test results; and

(e)   comparing the matrix of test results of said plurality of test, metabolically active whole cells in said step (d) with the matrix of reference results obtained from said plurality of reference, metabolically active whole cells in said step (b).

74.   The method of Claim 73, wherein said step (b) includes measuring an intensity of said fluorescent second state against time.

75.   The method of Claim 73, wherein said step (b) includes measuring a magnitude of said fluorescent second state at a point of time.

76.   The method of Claim 73, wherein said step (e), the matrix of test results and the matrix of reference results are compared to obtain a genetic analysis of the plurality of test, metabolically active whole cells.

77.   The method of Claim 73, wherein said step ( ) is performed by a computer.

78.   The method of Claim 73, said step (e) including performing a Non-Negative Least Squares technique on the matrix of test results and the matrix of reference results to classify the matrix of test results.

79.   The method of Claim 73, said step (e) including performing an Analysis of Variance technique on the matrix of test results and the matrix of reference results to classify the matrix of test results.

80.   The method of Claim 79, wherein the analysis of variance techniques includes determining a plurality of eigenvectors and eigenvalues.

81.   The method of Claim 73, said step (e) including performing an analysis of variance technique on the test results and the reference results to obtain a reduced set of test results and reference results.

82.   The method of Claim 81, said step (e) further including performing a Non-Negative Least Squares technique on the reduced set of test results and reference results to classify the test results.

83.   The method of Claim 81, said step (e) further including inputting the required set of test results and reference results to a Neural Network with back propagation to classify the test results.

84.   The method of Claim 81, said step (e), further including inputting the reduced set of test results and reference results to an expert system with Look-Up tables to classify the test results.

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85. The method of Claim 77, wherein the computer includes a Neural Network with back propagation for classifying the matrix of test results.

86. The method of Claim 77, wherein the computer includes an expert system with Look-Up tables for classifying the matrix of test results.

87. The method of Claim 73, wherein said step (e) compares the matrix of test results and the matrix of reference results to diagnose the plurality of test, metabolically active whole cells.

88. The method of Claim 73, wherein said step (e) compares the matrix of test results and the matrix of reference results to monitor enzyme activity representing morphology or cell type of the plurality of test, metabolically active whole cells.

89. The method of Claim 73, wherein said step (e) compares the matrix of test results and the matrix of reference results to monitor enzyme activity of the plurality of test, metabolically active whole cells over time.

90. The method of Claim 73, wherein said step (e) diagnoses the plurality of test, metabolically active whole cells for leukemia, wherein the at least one normally functioning enzymes include Pro, Lys, Gly, Ala, Gln-Ser, Thr-Pro, Val-Ser, Leu-Gly, and Gly-Pro and the plurality of test, metabolically active whole cells include lymphocyte cells and granulocyte cells.

91. A method of performing an assay for detecting the presence of a disease comprising:

(a) contacting a test, metabolically active whole cell with an assay reagent, said assay reagent containing at least one water soluble assay compound or water soluble physiologically acceptable salt thereof having a

fluorogenic indicator group and a leaving group, said leaving group being selected for cleavage by on of said at least one normally functioning enzyme the activity of which changes with the presence of the disease, said indicator group being in a non-fluorescent first state when bonded to said leaving group, and being in a fluorescent second state excitable at a wavelength above 450 nm when said leaving group is cleaved from said indicator group by the one of said at least one normally functioning enzyme for a period of time at least sufficient for said assay compound to be transferred into said cell and for said leaving group to be cleaved inside said cell from said indicator group by said enzyme;

(b)   sensing for said fluorescent second state of the indicator group for the test, metabolically active whole cell to produce a matrix of test results; and

(c)   comparing the matrix of test results of said test metabolically active whole cell with a matrix of reference results obtained from at least one of a diseased reference cell and a non-diseased reference cell.

92.   The method of Claim 91, wherein said step (b) includes measuring an intensity of said fluorescent second state against time.

93.   The method of Claim 91, wherein said step (b) includes measuring a magnitude of said fluorescent second state at a point of time.

94.   The method of Claim 91, wherein in said step (c), the test results and the reference results are compared to obtain a genetic analysis of the test, metabolically active whole cell.

95.   The method of Claim 91, wherein said step (c) is performed by a computer.

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96.   The method of Claim 91, said step (c) including performing a Non-Negative Least Squares technique on the matrix of test results and the matrix of reference results to classify the matrix of test results.

97.   The method of Claim 91, said step (c) including performing an Analysis of Variance technique on the matrix of test results and the matrix of reference results to classify the matrix of test results.

98.   The method of Claim 97, wherein the analysis of variance technique includes detecting a plurality of eigenvectors and eigenvalues.

99. The method of Claim 91, said step (c) including performing an analysis of variance technique on the test results and the reference results to obtain a reduced set of test results and reference results.

100.   The method of Claim 98, said step (c) further including performing a Non-Negative Least Squares technique on the reduced set of test results and reference results to classify the test results.

101.   The method of Claim 98, said step (c) further including inputting the reduced set of test results and reference results to a Neural Network with back propagation to classify the test results.

102.   The method of Claim 98, said step (c) further including inputting the reduced set of test results and reference results to an expert system with Look-Up tables to classify the test results.

103.   The method of Claim 95, wherein the computer includes a Neural Network with back propagation for classifying the matrix of test results.

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104.   The method of Claim 105, wherein the computer includes an expert system with Look-Up tables for classifying the matrix of test results.

105.   The method of Claim 91, wherein said step (c) compares the matrix of test results and the matrix of reference results to diagnose the test, metabolically active whole cell.

106.   The method of Claim 91, wherein said step (c) compares the matrix of test results and the matrix of reference results to monitor enzyme activity representing morphology or cell type of the test, metabolically active whole cell.

107.   The method of Claim 91, wherein said step (c) compares the matrix of test results and the matrix of reference results to monitor enzyme activity of the test, metabolically active whole cell over time.

108.   The method of Claim 91, wherein said step (c) diagnoses the test, metabolically active whole cell for leukemia, wherein the at least one normally functioning enzymes include Pro, Lys, Gly, Ala, Gln-Ser, Thr-Pro, Val-Ser, Leu-Gly, and Gly-Pro and the test, metabolically active whole cell is a lymphocyte cell or a granulocyte cell.

109.   The method of Claim 20, wherein cells are classified into cell types after determining the activity of said endogenous enzyme.

110.   The method of Claim 109, wherein enzymes are measured which distinguish nucleated red blood cells from non-nucleated red blood cells.

111.   The method of Claim 20, wherein cells obtained from a patient are tested for the presence of one or more of said endogenous enzymes to determine whether said patient has a disease or abnormal condition.

112. The method of Claim 20, wherein said disease or abnormal condition is selected from the group consisting of cancer, infection, sepsis, immune disorder and anemia.

113. The method of Claim 20, wherein said disease or abnormal condition which is measured or monitored is selected from the group consisting of hairy cell leukemia, chronic lymphocytic leukemia, B-cell leukemia, infection by bacteria, virus, fungus or yeast, sepsis, inflammatory conditions, corticosteroid treatment, AIDS, tuberculosis, metastatic potential of solid tumors, activation and/or modulation of macrophages, hereditary non-spherocytic hemolytic anemia, female reproductive tract cancer, breast tumor, colon cancer, Werthen's disease, pancreatic cancer, ovarian cancer, uterine cancer, cervical cancer, Gaucher's disease, Fabry disease, Tay Sach's, Wolman's disease, prostatic cancer, adenosine deaminase deficiency and lymphoma.

114. The method of Claim 20, wherein an infection selected from the group consisting of AIDS, cytomegalovirus (CMV), herpes, hepatitis, and syphilis is monitored.

115. The method of Claim 20, wherein a cell type selected from the group consisting of mature erythrocytes, nucleated erythrocytes, T-lymphocytes, B-lymphocytes, NK lymphs, atypical lymphs, eosinophils, lymphoid blasts, myeloid blasts, monocytic blasts, promonocytes, large mature monocytes, macrophages in a resting state, activated macrophages, elicited macrophages, basophils, promyelocyte, myelocyte, bands, mature neutrophils, platelets, neutrophil toxic granulation, neutrophil non-toxic granulation, platelet clumps, megakaryocytes, and reactive monocytes is determined.

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116. The method of Claim 76 or 94, which further comprises performing genetic analysis by PCR on test cells.

FIG. 1A

SAMPLE
(Leukocytes)

⇩

WASH 4X
> 10,000: 1

⇩

ADD
REAGENT
INCUBATE

⇩

LYSE
UNWANTED
CELLS

⇩

DATA
COLLECTION

⇩

RESULT

FIG. 1B

SAMPLE
(Leukocytes)

⇩

LYSE
UNWANTED
CELLS

⇩

WASH 4X

⇩

ADD
REAGENT
INCUBATE

⇩

DATA
COLLECTION

⇩

RESULT

FIG. 1C

SAMPLE
(Platelets,
RBCs &
Leukocytes)

⇩

WASH 4X

⇩

ADD
REAGENT
INCUBATE

⇩

DATA
COLLECTION

⇩

RESULT

FIG. 1D

SAMPLE
(Platelets or
RBCs or
Lymph, Mono,
Gran)

⇩

MECHANICAL
SEPARATION
OF CELL
POPULATION

⇩

WASH 4X

⇩

ADD
REAGENT
INCUBATE

⇩

DATA
COLLECTION

⇩

RESULT

FIG. 2A

FIG. 2B

FIG. 2C

FIG. 2D

RELATIVE ENZYMATIC ACTIVITY

pH

BLK 4.50 5.00 5.50 6.00 6.50 7.00 7.50 8.00 8.50 9.00

90 80 70 60 50 40 30 20 10 0

LG ACETATE ABN
LG Z ABN
LG TFA ABN
LG TRTRTE ABN

FIG. 3A

FIG. 3B

FIG. 3C

FIG. 3D

FIG. 4A

FIG. 4B

FIG.5A

FIG.5B

FIG.6A

FIG.6B

Leu–Gly   Leu–Gly–Leu–Gly   Leu–Leu–Arg

Gly–Leu   Thr–Pro   Gly–Leu

Normal
Breast

FIG.7

No Drug   0.0001μg/ml

0.001μg/ml   0.01μg/ml

0.1μg/ml   1.0μg/ml

FIG.8A

No drug

0.05μg/ml

0.5μg/ml

5μg/ml

FIG.8B

Lymphocyte

Peritoneal macrophage

Breast tumor

Infiltrating tumor macrophage

Breast tumor cells with infiltrating macrophages

Calibration standards

FIG.9

FIG. 10A

BACKGROUND
(IN THOUSANDS)

TIME (MONTHS)

FIG. 10B

FIG. 11A

FIG. 11B

FIG. 11C

FIG. 11D

FLUORESCENCE
(PHOTONS/SECOND)

TIME (MONTHS)

FIG. 11E

BACKGROUND
(IN THOUSANDS)

TIME (MONTHS)

FIG. 11F

FIG. 12A

FIG. 12B

FIG. 12C

TIME (MONTHS)

BACKGROUND
(IN THOUSANDS)

FIG. 12D

FLUORESCENCE
(PHOTONS/SECOND)

TIME (MONTHS)

FIG. 13A

FIG. 13B

FIG. 14A

ANALYSIS OF VARIANCE VIA EIGENVECTORS

FULL COVARIANCE DATA MATRIX
DIMENSIONS: n+1 COLUMNS, nc * m ROWS

		NORMAL	DISEASE 1	DISEASE 2	......	DISEASE n
CELL TYPE 1	ENZYME 1					
	ENZYME 2					
	....					
	ENZYME m					
CELL TYPE 2	ENZYME 1					
	ENZYME 2					
	....					
	ENZYME m					
		....	....	....	....	....
CELL TYPE i	ENZYME 1					
	ENZYME 2					
	....					
	ENZYME m					
		....	....	....	....	....
CELL TYPE nc	ENZYME 1					
	ENZYME 2					
	....					
	ENZYME m					

EIGENVECTOR ANALYSIS

EIGENVECTOR 1   ...   EIGENVECTOR 2

REDUCED COVARIANCE DATA MATRIX
OF STRONGLY CONTRIBUTING FACTORS

DIMENSIONS: n+1 COLUMNS, m1+m2+... mnc ROWS;
mi ≤ m, FOR ALL i

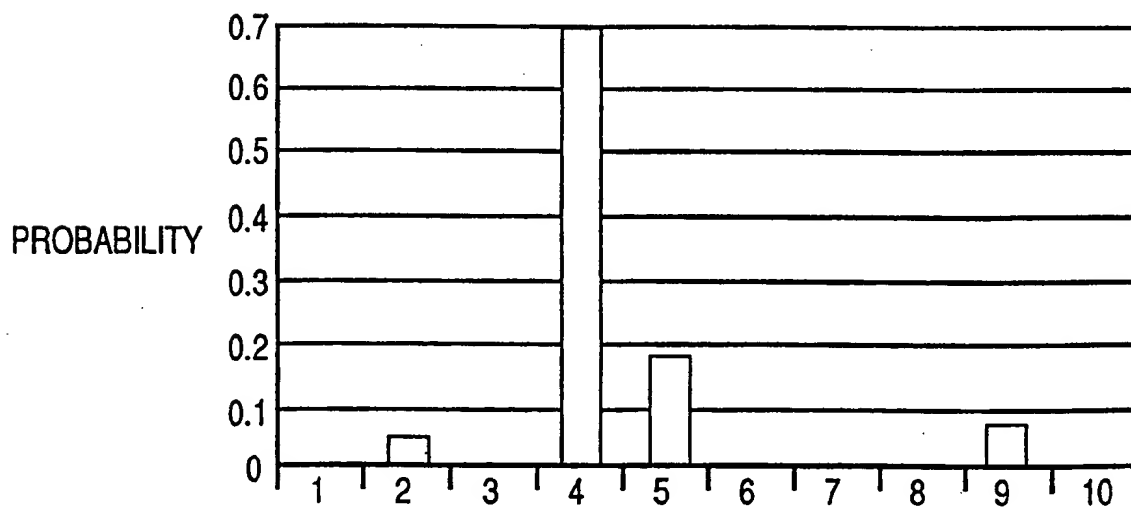|		NORMAL	DISEASE 1	DISEASE 2	......	DISEASE n
CELL TYPE 1	ENZYME 1					
	....					
	ENZYME m1					
CELL TYPE 2	ENZYME 1					
	....					
	ENZYME m2					
		....	....	....	....	....
CELL TYPE i	ENZYME 1					
	....					
	ENZYME mi					
		....	....	---	....	....
CELL TYPE nc	ENZYME 1					
	....					
	ENZYME mnc					

FIG. 14B
PREDICTION OF DISEASE STATE BY NNLS

REDUCED COVARIANCE DATA MATRIX
OF STRONGLY CONTRIBUTING FACTORS
DIMENSIONS: $n+1$ COLUMNS, $m1+m2+...$ mnc ROWS;
$mi < m$, FOR ALL i

		NORMAL	DISEASE 1	DISEASE 2	......	DISEASE n
CELL TYPE 1	ENZYME 1					
	....					
	ENZYME m1					
CELL TYPE 2	ENZYME 1					
	....					
	ENZYME m2					
		....	....	....	....	....
CELL TYPE i	ENZYME 1					
	....					
	ENZYME mi					
		....	....	....	....	....
CELL TYPE nc	ENZYME 1					
	....					
	ENZYME mnc					

↓

NNLS ANALYSIS

↓

PREDICTED DISEASE PROBABILITIES

PROBABILITY

DISEASE STATES

FIG. 15
NEURAL NETWORK FLOW CHART

CELL TYPE 1	NORMAL 1	NORMAL 2	NORMAL 3	DISEASE 1 PATIENT 1	PATIENT 2	PATIENT N	DISEASE n PATIENT 1	PATIENT 2	PATIENT n
ENZYME 1									
ENZYME 2									
ENZYME n									
CELL TYPE 2									
ENZYME 1a									
ENZYME 2a									
ENZYME na									
CELL TYPE x									
ENZYME 1x									
ENZYME 2x									
ENZYME nx									

TRAINING SET

SET THRESHOLD
LEARNING RATE
LEARNING MOMENTUM

TEST CASES
UNKNOWNS TO BE CLASSIFIED
AS DISEASE OR NORMAL

TIME COURSE
MONITORING OF INDIVIDUAL
PATIENT FOR RETURN TO
NORMALCY

APPLY ADVANCED
NEURAL NETWORK
PROGRAM PNN
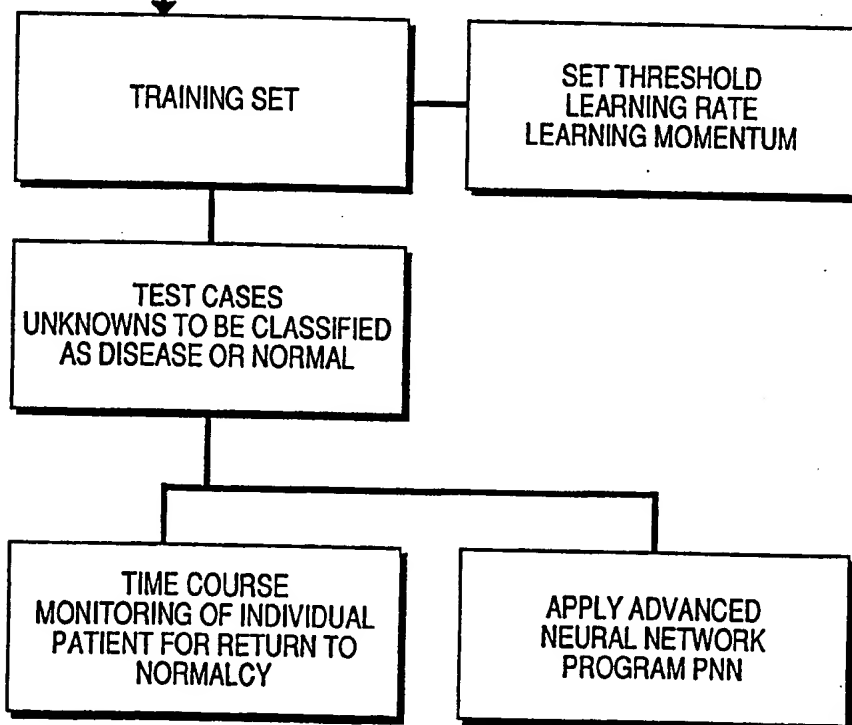
FIG. 16
SOLUTION USING FULL AND REDUCED MEASUREMENT

DISEASE STATE

RELATIVE AMPLITUDE

COLUMN 1 - FULL COVARIANCE DATA MATRIX
COLUMN 2 - EIGENVECTORS 1 AND 2
COLUMN 3 - EIGENVECTOR 1
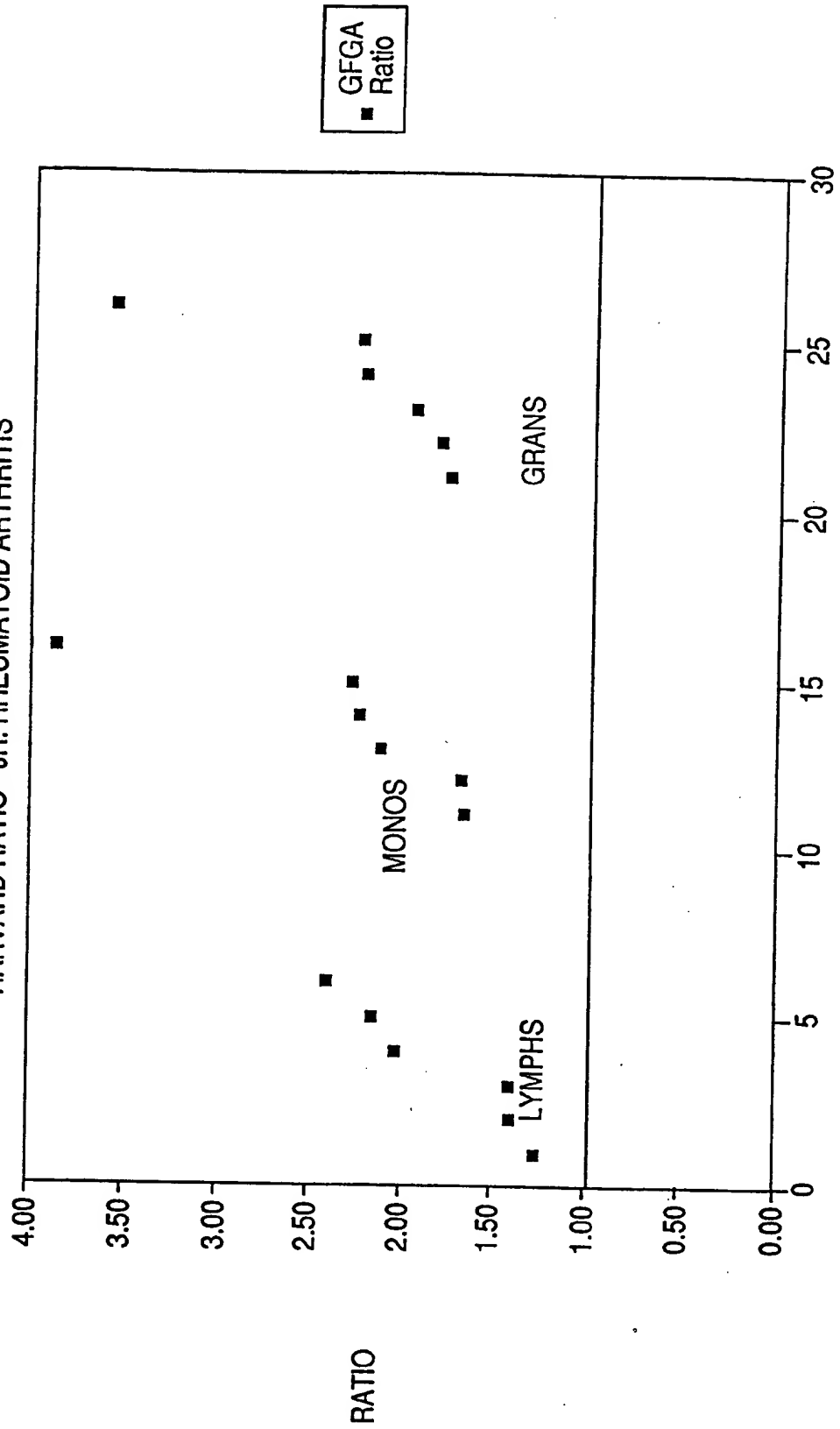COLUMN 4 - VARIANCE
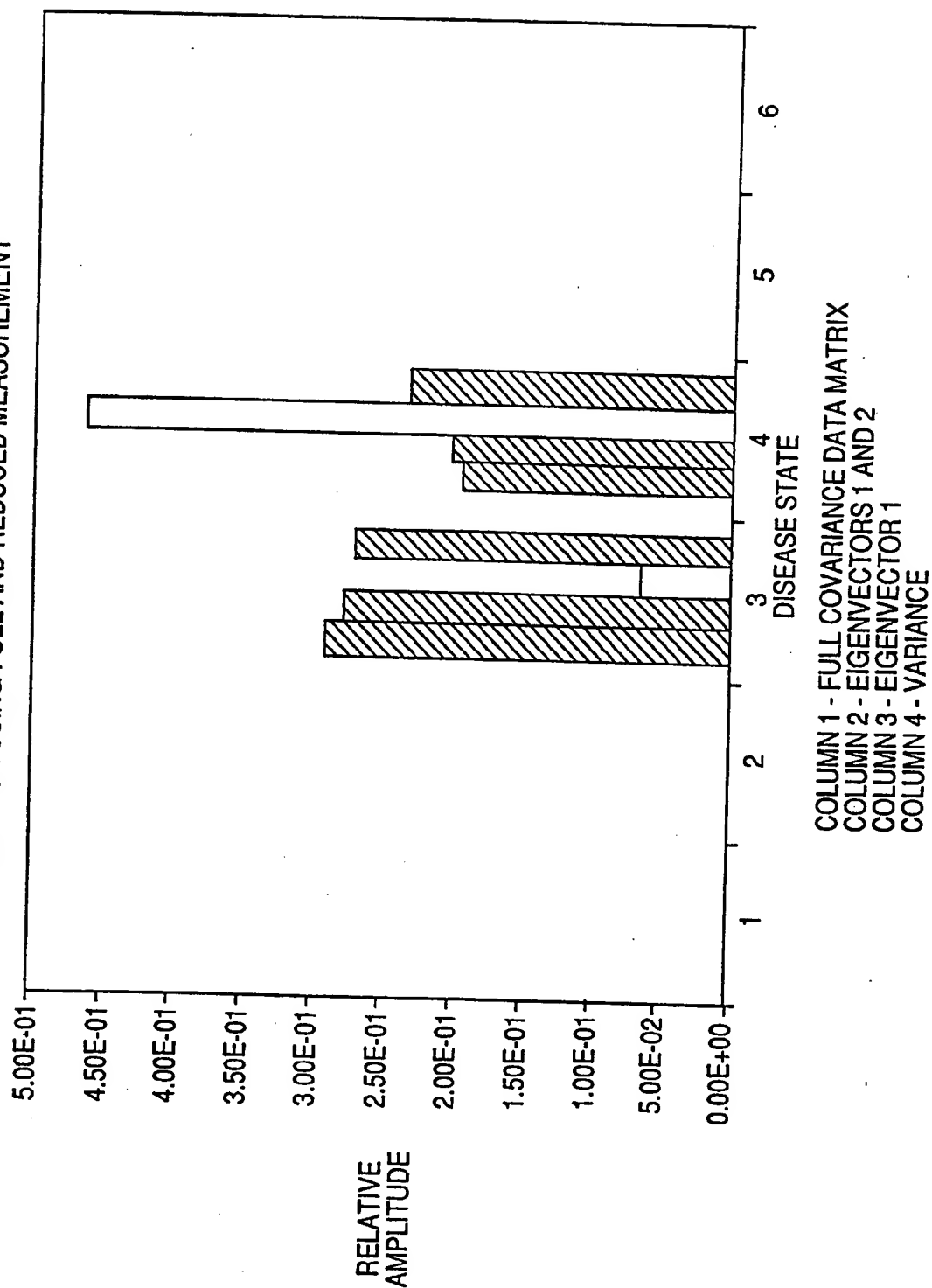
FIG. 17A
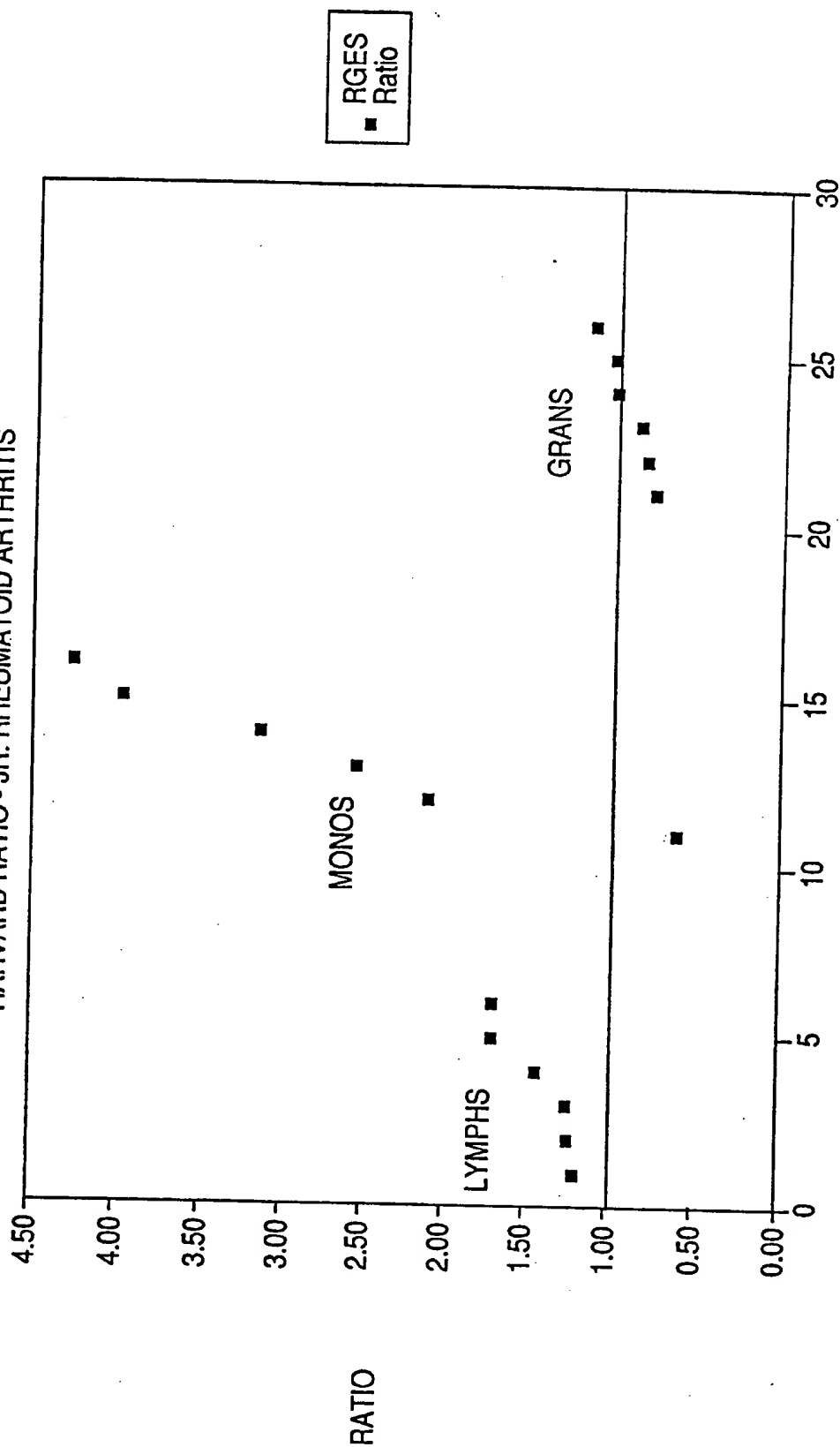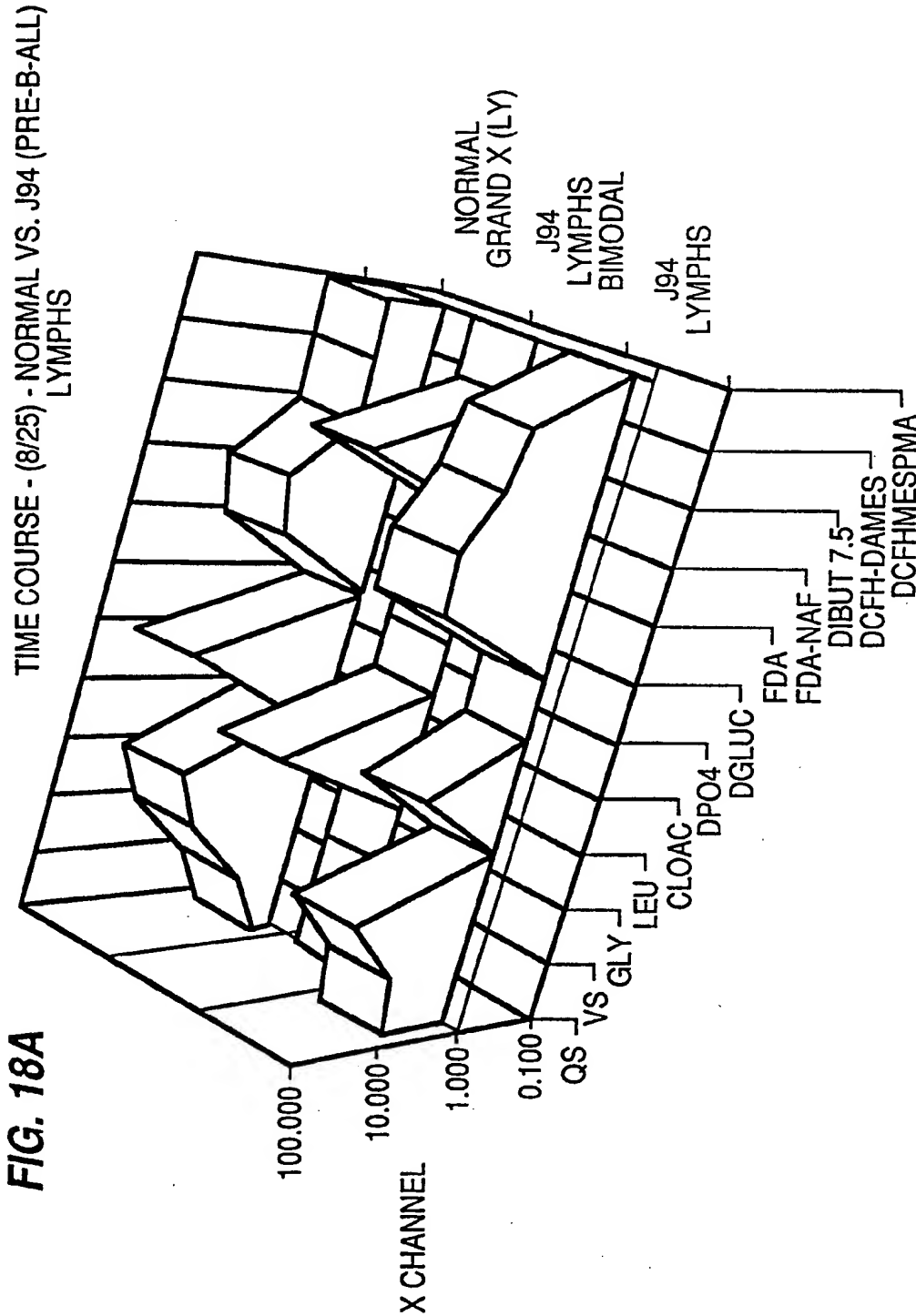HARVARD RATIO - JR. RHEUMATOID ARTHRITIS
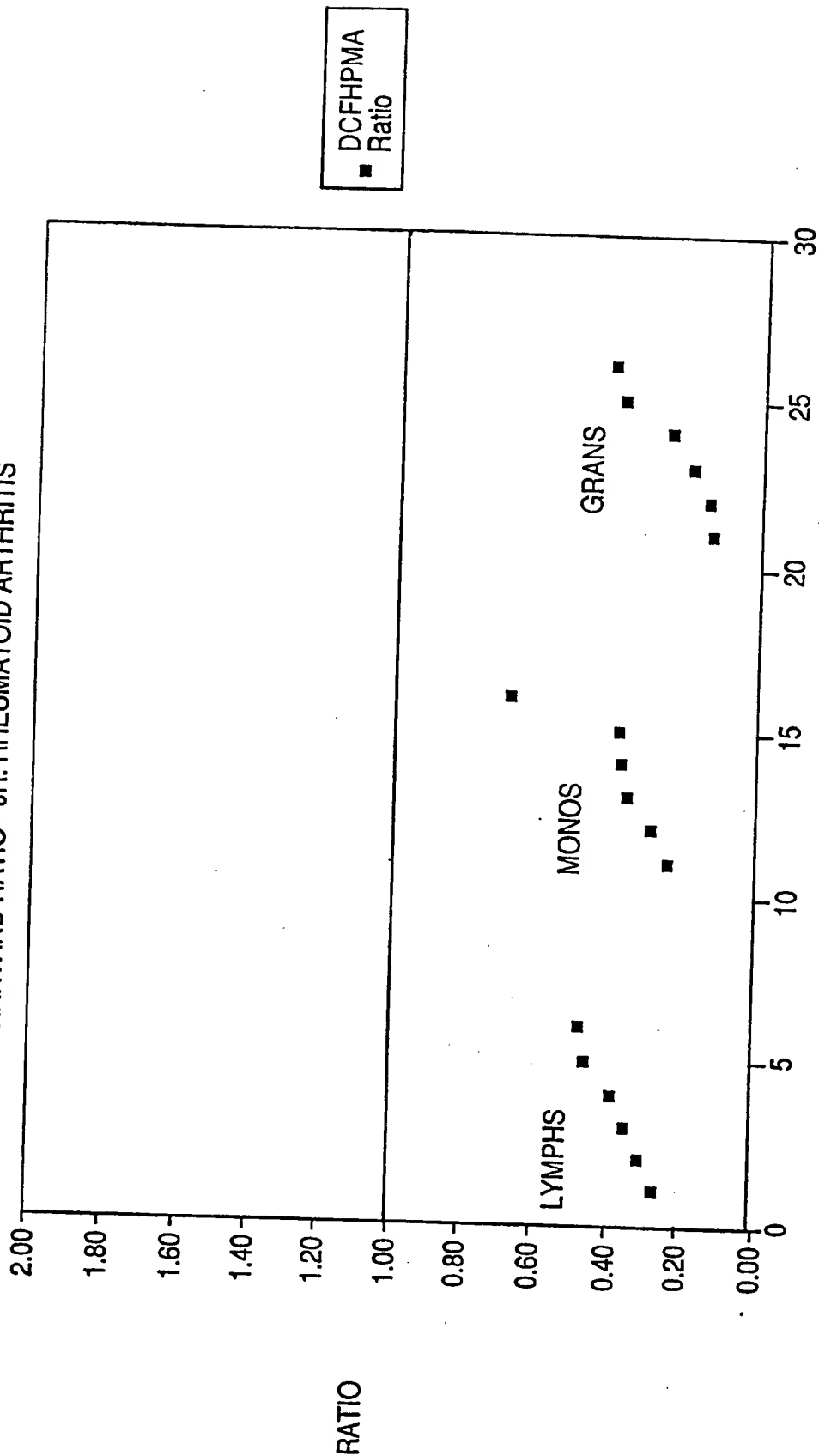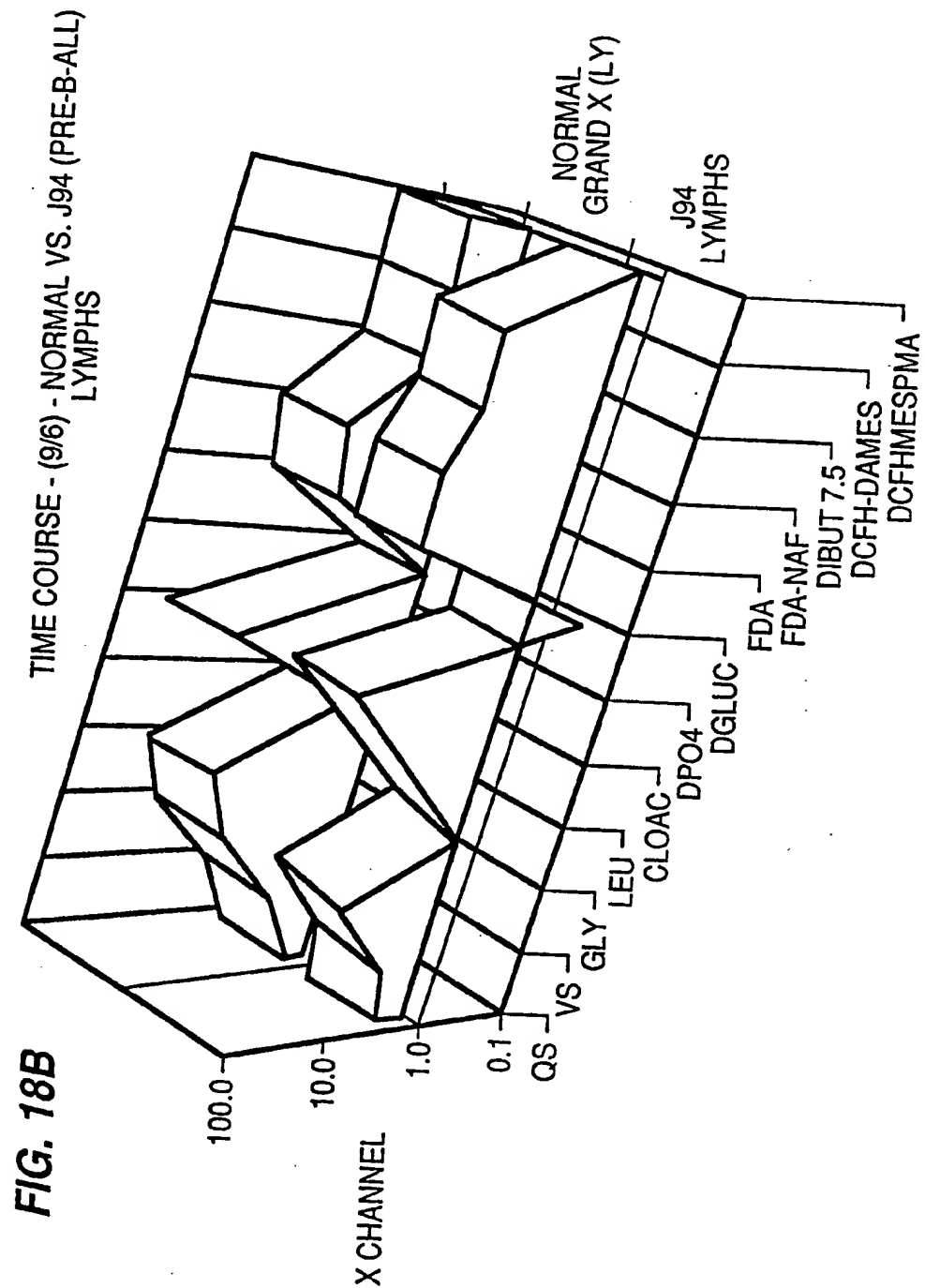
FIG. 17B

HARVARD RATIO - JR. RHEUMATOID ARTHRITIS

FIG. 17C

HARVARD RATIO - JR. RHEUMATOID ARTHRITIS

FIG. 18A

TIME COURSE - (8/25) - NORMAL VS. J94 (PRE-B-ALL) LYMPHS

FIG. 18B

TIME COURSE - (9/6) - NORMAL VS. J94 (PRE-B-ALL) LYMPHS

FIG. 18C

TIME COURSE - (9/21) - NORMAL VS. J94 (PRE-B-ALL) LYMPHS

FIG. 18D

TIME COURSE - (9/26) - NORMAL VS. J94 (PRE-B-ALL) LYMPHS

NORMAL GRAND X (LY)

J94 LYMPHS

100.0

10.0

1.0

0.1

X CHANNEL

FIG. 18E

TIME COURSE - (10/12) - NORMAL VS. J94 (PRE-B-ALL)
LYMPHS

FIG. 18F

TIME COURSE - (10/13) - NORMAL VS. J94 (PRE-B-ALL) LYMPHS

NORMAL GRAND X (LY)

J94 LYMPHS

X CHANNEL

100.0

10.0

1.0

0.1

FIG. 18G

SUBSTRATE	NORMAL GR MEAN — NORMAL GRAND X (LY)	PRIMARY PEAK — J94 8/25 J94 LYMPHS	PRIMARY PEAK — J94 9/21 J94 LYMPHS	PRIMARY PEAK — J94 10-12 J94 LYMPHS	BIMODAL PEAK — J94 8/25 J94 LYMPHS BIMODAL	BIMODAL PEAK — J94 9/21 J94 LYMPHS BIMODAL	BIMODAL PEAK — J94 10-12 J94 LYMPHS BIMODAL	PRIMARY PEAK — J94 9/6 J94 LYMPHS	PRIMARY PEAK — J94 9/26 J94 LYMPHS	PRIMARY PEAK — J94 10/13 J94 LYMPHS	BIMODAL PEAK — J94 9/6 J94 LYMPHS BIMODAL	BIMODAL PEAK — J94 9/26 J94 LYMPHS BIMODAL	BIMODAL PEAK — J94 10/13 J94 LYMPHS BIMODAL
QS	1.701	6.072		1.98				1.962					
VS	3.813	7.311		4.44			1.34	2.842					
GLY	18.178	30.120	24.880	11.91				11.380	22.760	12.23			
LEU	34.938			29.70						26.26			
CLOAC.	2.490	11.400	5.702	3.05	43.160	17.900		5.552	12.550	1.98			
DPO4	54.289			88.39				30.570					
DGLUC	0.941			1.87				0.247					
FDA	13.384	32.180	22.880	14.22				22.420	36.440	11.43			
FDA-NAF	17.980	33.790	24.500	12.43	34.480			22.360	27.230	11.50			
DIBUT 7.5	4.080	16.010	19.970	10.33				12.950	11.160	9.41			
DCFH-DAMES	4.107	11.850	10.83	3.89				14.640	25.040	3.07			
DCFHMESPMA	4.694			4.33						3.17			

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US,A,4 557 862 (MANGEL WALTER F ET AL) 10 December 1985	1-53, 55-71, 73-89, 91-107, 109-116
Y	see abstract; claims; examples see column 1, line 14 - line 19	
Y	--- WO,A,93 10461 (PROMEGA CORP) 27 May 1993	54,72, 90,108
X	see page 11 - page 14 see the whole document -----	54,72, 90,108 25

INTERNATIONAL SEARCH REPORT

Information on patent family members

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
US-A-5208148	04-05-93	NONE		
DE-A-1945663	18-03-71	DE-A-	2039999	17-02-72
		AT-A-	303268	15-10-72
		CH-A-	542445	15-11-73
		FR-A-	2070075	10-09-71
		US-A-	3786140	15-01-74
US-A-4557862	10-12-85	US-A-	4640893	03-02-87
WO-A-9310461	27-05-93	AU-A-	3129493	15-06-93
		CA-A-	2121842	27-05-93
		EP-A-	0646242	05-04-95
		JP-T-	7501444	16-02-95
		NO-A-	941781	29-06-94

International Application No
PCT/US 96/06860

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   C12Q1/34   G01N33/574

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6   C12Q   G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US,A,5 208 148 (HAUGLAND RICHARD P ET AL) 4 May 1993 see column 10, line 30 - column 11, last line see column 9, line 1 - column 10, line 29 see the whole document ---	1-24, 26-53
X	DE,A,19 45 663 (BOEHRINGER MANNHEIM GMBH) 18 March 1971 see the whole document ---	1-11
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search

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